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THE DISEASE OF POTATOES KNOWN AS "LEAK"[1]

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INTRODUCTION

The tuber-rot of potatoes (*Solanum tuberosum*) known as the "potato leak" is a disease of considerable importance in the delta region of the San Joaquin River, Cal. The rot is manifest in hot weather and appears soon after harvesting. As the potatoes in this region are sacked in the field and are practically all shipped immediately, the disease is therefore first evident in the car or warehouse. In extreme cases a whole shipment may be so badly damaged as to be worthless. If only a few "leakers" or "melters," as the rotten potatoes are called, are present it is usually necessary to sort the consignment. The cost of this sorting and the attendant shrinkage greatly increase the expense of production.

No exact data could be obtained as to the losses from this disease for any given season, but various estimates placed the damage for 1915 in the whole delta region, in which there were about 40,000 acres of potatoes, between $50,000 and $150,000. The general conditions and the methods of growing potatoes on these peat lands have been described by Orton (11),[3] Irish (8), and Shear (15). Orton and Shear have considered the diseases commonly found on potatoes in that region. In his paper Orton gives the results of a study of the potato leak, which is the only investigation of the disease heretofore reported. He was, however, prevented from completing the work on this disease to his satisfaction, and at his suggestion the writer took up the study.

In the study of the leak of potatoes described in the present paper it was planned to investigate further the causes of the disease, to study the organism or organisms causing it, their mode of entrance into the

[1] The work described in this article was carried out as a part of the potato-disease project of the Office of Cotton and Truck Disease Investigations.

[2] The writer's thanks are due Mr. W. V. Shear, of the Office of Horticultural and Pomological Investigations, for considerable assistance in the work at Stockton, Cal.

[3] Reference is made by number to "Literature cited," p. 639.

tuber, and, if possible, to obtain some data as to methods for its control. Part of the work was carried out at Stockton, Cal., and at various points in the delta potato fields near that city.

GENERAL APPEARANCE OF THE DISEASE

In taking up the study of the disease in the field, potatoes were examined at the sorting benches in the warehouses at Stockton, and various stages of the disease were observed. It was first apparent as a small brown discoloration around some wound, such as the wound made by the prong of a digging fork or by the breaking off of a "knob," which exposed the tissue of the inner part of the potato. The rot apparently did not affect tubers with unbroken skins. In the later stages of the disease the potatoes were brown over the entire surface, soft, and easily crushed. If sufficient pressure were applied to the tubers, a brownish watery liquid was exuded through breaks in the skin. Sacks containing potatoes in the advanced stages of this disease were frequently wet in patches where the rotten tubers had been crushed against the side. The interior of the rotten potato when broken was usually a dirty white, soon changing to a brown color around the edges. The center generally remained white for some time (Pl. XC).

ORGANISM CAUSING LEAK

RHIZOPUS NIGRICANS

Orton proved that the disease was caused by a fungus and concluded that the causal organism was *Rhizopus nigricans* Ehrenb. (10). He based his conclusions on the following premises: He observed a nonseptate mycelium in the rotted tubers and obtained *R. nigricans* in cultures made from these potatoes; he inoculated potatoes with this fungus and produced a rot similar in all appearances to leak.

That *R. nigricans* is able to rot Irish potatoes was also shown in unpublished studies by Mrs. Ethel Field Tillotson. In her experiments she used a strain of *R. nigricans* isolated from sweet potato (*Ipomoea batatas*). Her method of inoculation was to germinate the spores of the fungus in tubes of potato decoction and then pour the liquid, together with the germinated spores, into cavities in the potatoes. The inoculated tubers were placed in damp chambers, and in a few days the disease was evident.

With a strain of *R. nigricans* isolated from sweet potato by Mr. L. L. Harter the present writer was able to inoculate Irish potatoes successfully. The method developed by Mrs. Tillotson was followed in the earlier experiments. It was found unnecessary, however, to germinate the spores before inoculating the potatoes. Accordingly, in the later inoculations the tubers were inoculated directly from a culture of the fungus by inserting some of the spores and mycelium into rather deep wounds made in the tubers with a sterile knife. The inoculated potatoes were then placed in

a moist chamber and in from two to three days about 50 per cent showed evidences of the disease by brown coloration of the skin around the wound. In a week after inoculation the infected potatoes were usually entirely rotted. The skin was brown, and the interior of the potato was soft and watery. They were apparently typical leaky tubers. The fungus was readily reisolated from the rotten potatoes. From the investigations of Orton and the experiments of Mrs. Tillotson and of the present writer it is evident, then, that *R. nigricans* causes a rot of the Irish potato typical in appearance of the disease known as "leak." This work did not prove, however, that all cases of leak were due to *R. nigricans*, as it was very possible that other fungi acting in the same way might produce very similar results.

PYTHIUM DEBARYANUM

ISOLATION OF THE FUNGUS

Isolations of the fungus from potatoes were made by transferring portions of the partially rotted tubers obtained in the field to sterile tubes of slanted corn-meal agar and beef agar. In making these transfers the outer surface of the potato which had been washed in a 1 to 1,000 solution of mercuric chlorid was sliced away with a flamed knife and bits of the rotten portion of the potato farthest from the apparent point of infection were removed and placed in the culture tubes. In 24 hours a rather coarse hyalin mycelium was evident on the surface of the agar. After the cultures had grown for three days a microscopic examination of the fungus showed abundant fruiting bodies which much resembled the conidiospores of some species of Pythium. Occasionally structures were found which seemed to be oogonia and antheridia, though these were more frequently seen after a longer period. Transfers were made to the agar slants from 61 typical leaky tubers from a number of different fields. Of these transfers 49 proved to be cultures of this fungus, 5 of which were contaminated with bacteria. Six were cultures of bacteria only, and 6 were sterile. *R. nigricans* was not obtained in any of the cultures.

MORPHOLOGY OF THE FUNGUS

The fungus obtained from the leaky tubers was studied and found to be apparently a species of Pythium. The mycelium (fig. 1, *c*) of the fungus is rather coarse, irregularly branched, granular, usually nonseptate, though sometimes becoming septate when old. The conidia are borne either terminally or intercalarly. They are usually nearly spherical when mature and are from 12 to 26μ in diameter, averaging about 22μ. They germinate immediately with one or more germ tubes when they are placed in water at ordinary room temperatures (fig. 1, *d*). The oogonia are spherical and borne like the conidia either terminally or intercalarly.

They are from 15 to 25μ in diameter, averaging about 22μ. The antheridium (fig. 1, *b*) is borne either on the same filament as the oogonium or on an adjacent filament. If arising from the same filament it may be borne directly below the oogonium or some distance below. More than one antheridium was sometimes found attached to an oogonium. The oospores (fig. 1, *b*) are smooth, spherical, and thick-walled. They are from 14 to 19μ in diameter, average 16μ, and do not fill the oogonium. These measurements of the oogonia, oospores, and conidia all agree closely with those of *P. debaryanum*, as given by Butler. A culture of *P. debaryanum* used by Mr. C. P. Hartley in his studies on the damping-off of pine seedlings was obtained from the Office of Forest Pathology. This culture was a subculture of a strain which had been isolated from rotten

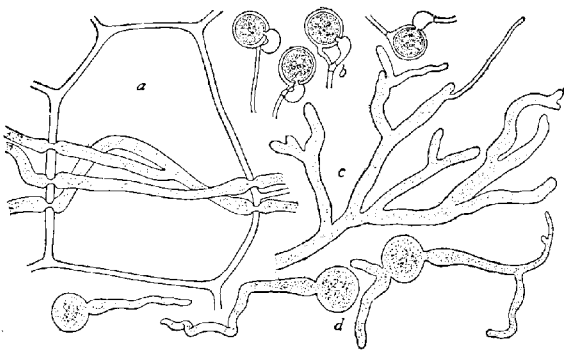


FIG. 1.—Microscopical appearance of *Pythium debaryanum* isolated from potatoes affected with potato leak: *a*, cell of a potato tuber showing fungus filaments therein; *b*, oogonia and antheridia; *c*, mycelium; *d*, germinating conidia.

potato by Edson (6) used by him in his studies on seedling diseases of sugar beets and then turned over to Hartley. This fungus agreed very closely with the *Pythium* sp. isolated from the leaky tubers in size of the conidia, oogonia, and oospores, habits of growth, and general appearance. Inoculated into potato tubers it produced a rot similar in all appearances to that produced by the fungus obtained from the leaky potatoes. It would seem, then, from the evidence above cited that the fungus isolated from the leaky potatoes in this study is the same as Hesse's *Pythium debaryanum* (7).

CULTURAL STUDIES

Cultures of the fungus were made on various kinds of media. The fungus grew well on beef, corn-meal, oatmeal, string-bean, Lima-bean, and potato agars, and Pfeffer's plant agar, potato plugs, and stems of *Melilotus alba*. Conidia and oogonia were formed when the fungus was

grown on string-bean and corn-meal agars, Pfeffer's plant agar, and the stems of *M. alba*. Neither conidia nor oogonia were found when the fungus was grown on the other kinds of media. The fungus produced both sexual and asexual reproductive bodies much more readily in Petri-dish cultures than in tubes. No sporangia or zoospores were seen in any of the cultures made in this study. It is of interest to note that Hesse (7), De Bary (2, 3), Sadebeck (13, 14), and Atkinson (1) are the only writers that to the author's knowledge record having observed the formation of zoospores by this fungus.

Cultures of the fungus were made from single conidia. To make these cultures some of the agar and mycelium from cultures which were producing conidia abundantly was ground up in sterile water. Corn-meal agar plates were poured in the usual way. The conidia germinated usually within an hour. The germinating spores were located by examining the inverted plates with a microscope. They were then marked and removed either to agar slants or to Petri dishes. The growth of these single-spore cultures was similar in all respects to that of the original 49 isolations of this fungus and to that of the strain of *P. debaryanum* obtained from the Office of Forest Pathology. They produced typical conidia, oogonia, and antheridia in abundance, and the mycelium showed the same characteristics as to branching and the granular structure of the protoplasm. Inoculations were made from these cultures into Burbank potatoes with positive results in 90 per cent of the cases. The fungus was reisolated from the rotted potatoes. The results obtained from these single-spore cultures indicate then that only the one fungus, *P. debaryanum*, was present in all the original 49 transfers.

The minimum, optimum, and maximum temperatures for growth and the temperatures at which growth was prevented were roughly determined for this fungus. For these experiments Petri-dish cultures on corn-meal agar were made from subcultures of five different isolations of the fungus and from the strain of *P. debaryanum* obtained from the Office of Forest Pathology. One Petri-dish culture for each constant temperature chamber was inoculated from subcultures from each isolation of the fungus. The growth of the cultures was measured each day for four days, after which the experiment was discontinued, as the culture media in some cases was entirely overgrown with mycelium.

The minimum temperature at which growth was noticeable in four days was between 5° and 8° C. No growth occurred at temperatures below 5°. The temperature at which growth is most rapid lies between 30° and 35°, and the maximum temperature at which growth can occur is between 35° and 40°. The fungus is killed at approximately 40°. Cultures from all five of the isolations from potatoes agreed as to these points, as also did the cultures from the strain of *P. debaryanum* obtained from the Office of Forest Pathology. The fungus was not killed at tem-

peratures below 5°, though growth was inhibited. The cultures from this chamber grew readily when placed in the incubator maintained at 30°. The experiments show that the range of temperature for growth is wide, about 30°, and that the optimum is high. Johnson (9) found the optimum temperature for growth of *P. debaryanum* to be 33°.

INOCULATION EXPERIMENTS

Inoculations were made into healthy California-grown Burbank potatoes from 30 of the 49 isolations of *P. debaryanum* obtained from diseased potatoes. Tubers were rotted and the fungus reisolated in all cases. Inoculations were also made with the bacterium which was sometimes obtained from the rotten tubers with no apparent effect. It seemed to be present as a saprophyte.

In the earlier inoculation experiments with *P. debaryanum*, the sterile tubers were inoculated with the fungus in wounds made with a flamed knife as in some of the experiments with *Rhizopus nigricans*. The inoculated tubers were then placed in moist chambers. Inasmuch as moist chambers, because of their limited volume of oxygen and their high humidity furnish rather abnormal conditions for the storing of potatoes, another method was developed in which the potatoes were maintained after inoculation under conditions which more nearly approached those found in storage. According to this method, the potatoes were disinfected as before and a small hole made in one side with a sterile knife. A ring, usually the ring of a Van Tieghem cell, was placed over the opening and cemented to the potato with petrolatum. A small quantity of sterile water was poured into the hole in the tuber and the inoculation made by placing some of the mycelium of the fungus in the water. A cover glass was then sealed on top of the cell with petrolatum. Various modifications of this method were tried to determine the size and depth of the wound necessary to insure a high percentage of successful inoculations. It was found that if the skin was removed from a small area of the potato which came within the ring when it was cemented in position and the inoculation made in a drop of sterile water on this wounded area, the results were as good as when deep wounds were made. Further experiments showed that it was sufficient to make a rather deep incision in the tuber with a sterile knife and introduce some mycelium to inoculate the potato successfully. The rots produced by such inoculations, however, became contaminated more frequently with bacteria than when the raw surface of the tuber was inclosed with a ring and cover glass. Numerous controls were prepared by cementing the ring to the unbroken surface of the tuber and placing therein some bits of mycelium in sterile water; also by pouring sterile water into wounds in the potatoes and sealing them as in the inoculation experiments. In none of these controls was there any infection.

In the inoculation experiments 210 sound potatoes of the Burbank variety were used, of which 177, or 84 per cent, were rotted.

Besides the experiments with California-grown potatoes, inoculation experiments were carried out with several eastern-grown varieties. These potatoes were kindly furnished by the Office of Horticulture and Pomological Investigations in most cases. The tubers were inoculated in deep wounds inclosed with a ring and cover glass, according to the method already described. Five different isolations of the fungus were used with each variety of potatoes. After inoculation they were placed in an incubator maintained at 30° C. and left there throughout the experiment. The results of this experiment are shown in Table I.

TABLE I.—*Results of inoculating several varieties of eastern-grown potatoes with Pythium debaryanum, as shown by the number of potatoes of each variety rotted*

Variety.	Number inoculated.	Number rotted.	Variety.	Number inoculated.	Number rotted.
Rose 4 (Florida) [1]	17	13	Early Ohio	11	10
Rose 4	9	0	Rural New York	11	7
Early Rose	12	5	Irish Cobbler	12	5
Triumph	9	5	Pearl	12	3
Green Mountain	10	7			

[1] Furnished by Mr. W. B. Clark, of the Office of Cotton and Truck Disease Investigations.

From the results shown in Table I it is evident that some of the varieties of eastern potatoes are about as susceptible to this disease as California-grown Burbanks used in the experiments already described. Early Ohio was apparently most susceptible, in that 10 potatoes rotted out of 11 inoculated. The other varieties seemed somewhat more resistant to this disease.

Inoculations were also made, using potatoes of undetermined varieties purchased in the Washington markets. A fair percentage of these inoculations were successful in all cases. It would seem, then, that susceptibility to this disease is not necessarily confined to potatoes grown on the peat lands of California.

Another series of inoculation experiments was carried out to ascertain what temperatures were most favorable for the growth of the fungus in the potato and at what temperatures no infection would result from an inoculation. In these experiments inoculated potatoes were kept at seven different temperatures, varying in 5-degree intervals from 5° to 35° C. Seventy potatoes of the Burbank variety were used. Forty of these potatoes, those intended for the lower temperatures, were kept in the ice box at about 10° C. for 24 hours before inoculation, so that their temperature at the time of inoculation would be more nearly that at which they were to be maintained during the experiments. For the inoculations

subcultures from five separate isolations of the fungus were used, and 14 potatoes were inoculated from subcultures from each isolation, 2 for each of the constant-temperature chambers. They were maintained at constant temperatures for one week and were then removed and examined. The results of these experiments are shown in Table II.

TABLE II.—*Results of experiments in which inoculated potatoes were maintained at constant temperatures, 10 Burbank potatoes in each chamber, maintained at constant temperature for one week. All sound potatoes were then placed in the 30° chamber for three days*

Temperature.	Number of tubers showing infection in one week.	Number of tubers which did not show evidences of infection in chambers originally used but which were rotted three days after removal to 30° chamber.	Total number of rotted potatoes.
°C.			
5	0	7	7
10	3	2	5
15	7	2	8
20	8	0	8
25	9	0	9
30	10	0	10
35	10	0	10

The results given in Table II show that a higher percentage of inoculated potatoes are rotted at temperatures near the optimum for growth of the fungus in artificial culture media than at the lower temperatures. It is evident, however, that temperatures near this optimum are not necessary for infection. As was to be expected, no rot was produced while the inoculated potatoes were maintained at 5° C., but when these potatoes were moved from this chamber to the incubator maintained at 30°, 70 per cent of them were rotted in three days. The growth of the fungus is apparently inhibited at the low temperature, but begins as soon as the temperature is raised. The lowest total amount of rot was in the potatoes maintained at 10° for the week. In this case 50 per cent of the inoculated potatoes rotted. The growth of the fungus in the potato is slower at the lower temperatures, 10° and 15°, than at the higher temperatures, as was found to be the case with this fungus on artificial-culture media.

It is evident from these experiments in which *P. debaryanum* was isolated from 49 diseased tubers, inoculations made from 30 of these isolations into healthy tubers, the disease produced, and the fungus subsequently reisolated that this fungus is frequently present in potatoes affected with leak and that when inoculated into the tubers, it causes this rot.

GROWTH OF PYTHIUM DEBARYANUM IN THE TUBER

The rate of growth of the fungus in the potato was approximately determined. A Green Mountain potato which had been inoculated in the usual way and allowed to remain at 30° C. for 67 hours was sliced open. The fungus was found to have penetrated to a depth of 4 cm. from the point of inoculation during this time. The average diameter of the cell of the potato, obtained by measuring a large number of cells, was found to be 138.7μ. By calculation the fungus must have passed through approximately 288 cells in 67 hours, or at the rate of 1 cell every 14 minutes. This calculation does not take into account the period of readjustment of the fungus before it begins to grow into the tissue of the potato, which is probably appreciable.

Portions of a potato tuber which had been rotted with *P. debaryanum* were killed, embedded in paraffin, sectioned, and stained.[1] Examination of these sections showed that the mycelium was distributed quite generally throughout the tissue of the host. It usually passes directly through the cell wall (fig. 1, *a*) and through the lumen of the cell, though it was found occasionally between the cells. It branches frequently. Where the hypha of the fungus passes through the cell wall, it is markedly constricted (fig. 1, *a*). Ward (16), in his work on this fungus, also observed that the opening made in the cell wall was smaller than the mean diameter of the fungus hyphæ. Rosenbaum (12) shows the same relation between cell wall of host plant and fungus hypha in his work with *Phytophthora cactorum* on ginseng.

INFECTION OF POTATOES FROM SOIL

It was mentioned earlier in this paper that the disease was observed only in potatoes which had been wounded. In inoculation experiments it was never possible to cause the disease without first breaking the skin of the potato. The wounds observed in the rotting potatoes in the field studies had been made when the potatoes were harvested, which leads to the conclusion that the organisms causing the leak are probably present in the soil and are introduced into the freshly wounded potato in digging. To obtain evidence on this point, Petri-dish cultures on corn-meal agar were made from samples of the peat soils from various parts of the delta potato region. *P. debaryanum* was found in every case. Inoculations were made by inserting some of the soil into holes in the tubers and in about 50 per cent of the cases the tubers were rotted. *P. debaryanum* was isolated from the rotted tubers.

Field tests were made on the effect of wounding the potatoes in digging. In these experiments seven sacks, or about 12 bushels, of potatoes were harvested. The work was done rather carelessly so that many tubers were injured with the digging forks. The sound potatoes were

[1] The writer is indebted to Mr. Charles S. Ridgway, of the Office of Tobacco Investigations, for the making and staining of these slides. They were stained in methylene blue-eosin combination which leaves the fungus hyphæ bright blue and the cell walls of the host plant red.

sorted out and sacked separately, and all the potatoes were stored in sacks in a warehouse under about the usual commercial conditions. The potatoes were sorted four days later and 65 diseased tubers were found, all of which had been wounded. They were sorted a second time eight days after digging and 52 more rotten tubers were found. None of the unwounded potatoes showed evidences of the disease at any time, and no more of the wounded tubers were rotten when they were sorted for the last time 15 days after harvesting. Transfers were made from some of these rotten tubers to corn-meal agar slants and *P. debaryanum* was obtained in all these cultures. It is evident that this fungus is generally present in these peat soils, that inoculations may be made by inserting some of the soil in wounds in the tubers, and that potatoes wounded in digging frequently become infected. Unwounded tubers are apparently not affected with this disease. It would seem probable from these experiments that more care in harvesting and sorting out of potatoes injured in digging would decrease the losses from this disease.

OTHER ROTS SOMETIMES MISTAKEN FOR LEAK

It is quite possible that tuber-rots produced by other fungi may be mistaken for potato leak. Two species of Fusarium, *F. radicicola* Wollenw. and *F. oxysporum* Schlecht., which produce tuber-rots of the potato are quite common in the San Joaquin potato region. Carpenter (5) has shown that either one or the other of these fungi is usually present in the jelly-end rot of potato tubers. He has also obtained *F. radicicola* from specimens of rotten potatoes from San Joaquin County, California, which were supposed to be affected with leak. The present writer has found rotten tubers in consignments of potatoes from California which had stood in the laboratory for a few weeks. These potatoes were apparently sound upon arrival, with the exception of a few which had small rotten spots in the stem end. At the end of a few weeks some of the tubers were entirely rotten and very much resembled those in the advanced stages of leak. *F. radicicola* was obtained from several such specimens. Neither *Pythium debaryanum* nor *Rhizopus nigricans* was ever obtained. Potato dealers at Stockton and potato growers say that the leak may develop after the potatoes have been in storage for a time and sometimes after they have been sorted. Under such conditions the rot is apparently not due to *P. debaryanum* nor *R. nigricans*, but to some other organism, probably a species of Fusarium in many instances, as in these experiments rots caused by *P. debaryanum* or *R. nigricans* were usually evident in three or four days. If an inoculated potato was sound at the end of a week it was not infected and the potato would remain sound indefinitely. The experiments in which potatoes were kept at low temperatures are, of course, excepted. It seems quite probable then that potatoes affected with rots caused by *Fusarium* spp. are sometimes confused with those affected with leak.

DISCUSSION OF RESULTS

It is evident from the experiments described in this paper and from the work that has been done heretofore that *R. nigricans* rots potato tubers. That it is the cause of a rot of potatoes under field and warehouse conditions has been shown by Orton (10). From the experiments carried out in this study, however, it seems that potato leak is most commonly caused by *P. debaryanum*. At least this seems to have been the case during the season of 1915.

When inoculated into potatoes, both fungi rot the tubers either very rapidly or not at all. It seems that if the disease is not well advanced in a week at 30° C. the potato is not infected. The rots produced by these fungi have practically the same general appearance.

The parasitism of *P. debaryanum* on seedlings of various plants is too well known to require discussion here. That it should be the cause of a potato disease of considerable importance is not surprising when the work of earlier writers is taken into account. Sadebeck (14), in 1875, reported the discovery of a species of Pythium parasitic upon potato plants near Coblenz. He considered the fungus to be *P. equiseti* Sadebeck. He mentions finding it on various parts of the plants. That *P. equiseti* was identical with *P. debaryanum* was later pointed out by De Bary (2). De Bary in some of his experiments grew *P. debaryanum* on living potato tubers. Ward (16) also cultivated it on this host and considered potatoes ". . . a very good medium for the cultivation of the fungus." Edson (6) recently obtained this fungus from rotten potato tubers. No one seems to have succeeded in inoculating any part of the potato plant except the tuber with this organism.

That this fungus should cause so much damage to potatoes in the San Joaquin delta region is probably largely due to the conditions and methods of handling the potatoes in that section. As has been said, the potatoes are dug with forks, and many are wounded in the process. Potatoes with branches, or "knobs," are quite common, and these branches are usually broken off in harvesting, if the potato is of marketable size, and the main tuber retained. Perhaps the broken surface of the tuber is rubbed in the soil, "to dry it." That these are excellent methods for inoculating potatoes with *P. debaryanum* has been shown. The potatoes are sacked as soon as dug. They may then stand in the sun for some hours before they are hauled to the car or boat landing for shipment. In the car or on the boat the sacks are usually piled up. The humidity among these tubers is, of course, high because of the high rate of transpiration. This, together with the relatively high temperature, offers good conditions for the development of any parasitic fungus, such as *P. debaryanum*, with which the tubers may have been inoculated. It is quite possible that the leak of potatoes would have been reported from other localities where either *R. nigricans* or *P. debaryanum* are common in the

soils if the methods of harvesting and handling and the temperature conditions were as favorable for the development of these parasites as they are in the delta region of the San Joaquin River.

It is considered by the potato growers of this region that the disease is much more common in hot weather. In these experiments it was shown that the optimum temperature for growth of the fungus is high (between 30° and 35° C.) and that the fungus infects the potatoes more readily at temperatures near this optimum. At the lower temperatures the percentage of infection is not so high, and the growth of the fungus is retarded or, as in the case of the experiments at 5°, inhibited while the potatoes remained at that temperature. It would seem then that lowering the temperatures of the cars and storage warehouse might retard the development of the disease, but that the infected potatoes would rot as soon as the temperature was raised. From the data now at hand, icing the cars and cold storage of the potatoes would seem to be of doubtful value as control measures. The control of the disease seems more likely to lie along the lines of better methods of harvesting and handling, as Orton suggested (10), and a careful sorting out of all wounded tubers.

CONCLUSIONS

In the work described in this paper the conclusion of Orton that *Rhizopus nigricans* Ehrenb. can cause a rot of potatoes has been corroborated. This fungus was not, however, isolated in the field experiments from tubers affected with leak. A fungus was obtained 49 times in 61 attempts. The cultures were made from a different tuber each time. A study was made of the fungus and it was found to be *Pythium debaryanum* Hesse. In inoculation experiments this fungus produced a rot typical in all appearances to the potato leak, and was readily reisolated from the diseased tuber. It seems probable that the disease is produced by both *R. nigricans* and *P. debaryanum*. The latter is apparently more frequently the causal organism.

P. debaryanum was found in soil samples taken from various parts of the delta potato region. The disease was produced by inserting some of this soil in wounds in the potato tubers and *P. debaryanum* was isolated from these rotted potatoes. Infection apparently takes place in the field by some of this infected soil getting into wounds made in digging. No cases of infection were observed either under field conditions or in the laboratory where the skin of the tuber was unbroken. From the results of these experiments it seems that the disease might be controlled by more care in harvesting and handling the potatoes and a careful sorting out of all wounded tubers.

LITERATURE CITED

(1) ATKINSON, G. F.
1894. Preliminary note on the swarm spores of Pythium and Ceratiomyxa. *In* Bot. Gaz., v. 19, p. 375–378.

(2) BARY, Anton de.
1881. Untersuchungen über die Peronosporeen und Saprolegnieen und die Grundlagen eines natürlichen Systems der Pilze. *In* Abhandl. Senckenb. Naturf. Gesell., Bd. 12, Heft 3/4, p. 225–370, 6 pl.

(3) ———
1881. Zur Kenntniss der Peronosporeen. *In* Bot. Ztg., Jahrg. 39, No. 33, p. 521–530, pl. 5; No. 34, p. 537–543; No. 35, p. 553–563; No. 36, p. 569–578; No. 37, p. 585–595; No. 38, p. 601–609; No. 39, p. 617–625.

(4) BUTLER, E. J.
1907. An account of the genus Pythium and some Chytridiaceæ. *In* Mem. Dept. Agr. India, Bot. Ser., v. 1, no. 5, p. 1–160, 10 pl.

(5) CARPENTER, C. W.
1915. Some potato tuber-rots caused by species of Fusarium. *In* Jour. Agr. Research, v. 5, no. 5, p. 183–210, pl. A–B (col.), 14–19. Literature cited, p. 208–209.

(6) EDSON, H. A.
1915. Seedling diseases of sugar beets and their relation to root-rot and crown-rot. *In* Jour. Agr. Research, v. 4, no. 2, p. 135–168, pl. 16–26.

(7) HESSE, Rudolph.
1874. Pythium de Baryanum ein endophytischer Schmarotzer in den Geweben der Keimlinge der Leindotter, der Rüben, des Spergels und einiger anderer landwirtschaftlichen Kulturpflanzen. 76 p., 2 pl. Halle a/S. Inaugural-Dissertation.

(8) IRISH, John P., jr.
1913. The work of the Delta experiment farm in 1912. *In* U. S. Dept. Agr. Bur. Plant Indus. Circ. 127, p. 3–13, 1 fig.

(9) JOHNSON, James.
1914. The control of damping off disease in plant beds. Wis. Agr. Exp. Sta. Research Bul. 31, p. 29–61, illus. Bibliography, p. 60–61.

(10) ORTON, W. A.
1909. Decay of potatoes due to Rhizopus nigricans. (Abstract.) *In* Science, n. s. v. 29, no. 753, p. 916.

(11) ———
1909. Potato diseases in San Joaquin County, California. U. S. Dept. Agr. Bur. Plant Indus. Circ. 23, 14 p.

(12) ROSENBAUM, Joseph.
1915. Phytophthora disease of ginseng. N. Y. Cornell Agr. Exp. Sta. Bul. 363, p. 61–106, fig. 2–18.

(13) SADEBECK, R.
1874. [Ueber Pythium equiseti.] *In* Verhandl. Bot. Ver. Brandenb., Jahrg. 16, p. 116–126.

(14) ———
1875. [Ueber Pythium equiseti und dessen Infectionskraft für die Kartoffelpflanze.] *In* Sitzber. Gesell. Naturf. Freunde Berlin, Jahrg. 1875, p. 148–151.

(15) SHEAR, W. V.
1914. Potato growing in the San Joaquin and Sacramento deltas of California. Cal. Agr. Exp. Sta. Circ. 120, 11 p., 7 fig.

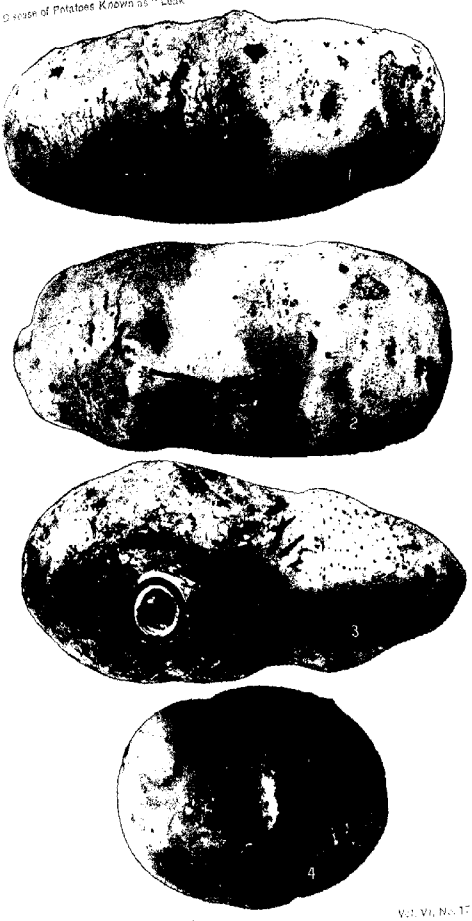
(16) WARD, H. M.
1883. Observations on the genus Pythium (Pringsh.). *In* Quart. Jour. Micros. Sci., n. s. v. 23, p. 485–515, pl. 34–36.

PLATE XC

Potatoes affected with potato leak:

Fig. 1, 2.—Natural infection from fork wound; photographed by Dr. W. A. Orton.
Fig. 3.—Rot produced by inoculation with *Pythium debaryanum*.
Fig. 4.—Rot produced by inoculation with *Rhizopus nigricans*. Inoculation made by Mrs. Tillotson.

Disease of Potatoes Known as "Leak"



DIGESTIBILITY OF HARD PALATES OF CATTLE

By C. F. LANGWORTHY, *Chief*, and A. D. HOLMES, *Scientific Assistant, Office of Home Economics, States Relations Service*

The so-called "hard palates," which are taken from the roof of the mouth of beef animals, have not in the past been utilized to any extent as food. They contain very little muscular tissue, such as is characteristic of meats in general, and possess a ribbed outer surface that is black or white in color, very rough, and of an unattractive appearance.

The microscopic examination of the structural constituents of hard palates of cattle reveals a stratified layer of epithelium which is in a state of cornification. The extent of this layer is possibly one-sixteenth of the entire thickness. The connective tissue portion of the mucous membrane consists of a dense feltwork of white fibrous tissue arranged in dense interlacing bundles; the individual fibers of the bundles, comprising about 60 per cent, are matted together as closely as in tendon tissue or sinews and are interwoven with about 20 per cent of elastic (erectile) fibers, 10 per cent of involuntary muscle, and about 10 per cent of looser fibrous tissue attaching the mucous membrane to the periosteum. This looser tissue contains a small amount of fat and very few glands.

A chemical examination of hard palates showed that when freshly procured they have the following composition: Water, 71.0 per cent; protein (N×6.25), 22.2 per cent (or protein by difference, 16.6 per cent); fat, 11.8 per cent; and ash, 0.6 per cent. The high protein content suggested that this material might be of value for food. Since little, if any, experimental evidence is available regarding the thoroughness of digestion of such tissue when eaten in quantity, a number of experiments were undertaken at the suggestion of the Bureau of Animal Industry to determine the digestibility of hard palates by human subjects. This means for practical purposes the digestibility of the nitrogenous material present, since the proportion of fat supplied by the cooked hard palates is small.

COOKING HARD PALATES

The material for study was obtained from a local abattoir and supplied to the Office of Home Economics by the Bureau of Animal Industry. Before the digestion experiments could be undertaken, it was necessary to find some way of cooking and serving the hard palates which would make it possible to eat them in quantity. At first the attempt was made to put the raw material through an ordinary household meat cutter with the idea that it might then be fried in small cakes, like Hamburg steak, but the material was so firm and tough that it

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could not be minced in this way. Accordingly it was decided to cook the palates before trying to mince them, and tests showed that after boiling for two or three hours they could be easily minced with a meat cutter and that so prepared the texture as well as the flavor was not disagreeable, particularly if the palates were combined with other food materials.

The average composition of the cooked palates was found to be as follows: Water, 71.1 per cent; protein (N×6.25), 21.8 per cent (or protein by difference, 22.3 per cent); fat, 6.3 per cent; and ash, 0.3 per cent. The material used for analysis weighed before cooking 15¼ ounces and after cooking 14 ounces, the total loss therefore being only 1½ ounces. As will be noted by referring to the percentage composition of the raw material, the boiled palates had, in round numbers, only one-half the fat, one-half the ash, and nine-tenths the protein content of the fresh material. As found by analysis, 50.0 per cent of the ash, 46.6 per cent of the fat, and 11.3 per cent of the protein originally present were removed by cooking. In general, the observed effects are in accord with Grindley's observations[1] that, except for a lowered fat and ash content and the removal of some soluble nitrogenous material, cooked meat has very much the same proximate composition as it has raw.

The water in which the palates were boiled did not look at all like that in which beef is cooked, but was white in color and not unlike milk in appearance. The character of the nitrogenous constituents present was not studied in detail, but preliminary tests indicated that gelatin predominated, with traces of coagulable albumin, globulin, and primary proteoses.

Some attention was given to the hard palate fat which floated to the top of the liquor in which the palates were boiled. This hardened on cooling and was purified by remelting several times to remove the sediment. The product had a deep-yellow color, a mild flavor, and an appearance suggesting butter, though rather more granular. It was found to have a melting point of 34° C., an iodin number of 52.53, and a refractive index of 1.4586.[2] The amount obtained was not sufficient for further study.

The cooked palates had a mild and not unpleasant flavor and in appearance resembled cooked gristle or connective tissue rather than lean meat, this resemblance being noticeable even when the material was finely ground. It was apparent that the cooked palates would be much more acceptable as the principal constituent of the experimental ration if prepared in some savory form, and meat cakes and meat loaf naturally suggested themselves as possibilities. The meat cakes

[1] Grindley, H. S., and Mojonnier, Timothy. Experiments on losses in cooking meat, 1900–1903. U. S. Dept. Agr. Office Exp. Stas. Bul. 141, p. 94. 1904.

[2] Information regarding the structure and composition of the hard palates and the chemical nature of the material extracted during cooking was supplied by the Bureau of Animal Industry.

did not prove satisfactory, having, when thoroughly cooked and well browned, a flavor suggesting that of scorched or burned gristle or bone. On the other hand, meat loaf made according to a common household recipe and containing in addition to the hard palates some flour, butter, and onions, and sweet herb, salt, and pepper as seasoning was found to be satisfactory for the purpose. The flour served to bind the material together so that the loaf would retain its shape and could be sliced without crumbling, while the butter improved both the texture and the flavor.

EXPERIMENTAL RATION

Experience has shown that the normal individual eats more heartily of a food material if it forms a part of a mixed ration than if it is the only food served for several successive meals. Accordingly, with the meat loaf made from hard palates, a uniform basal ration simple in character (crackers and butter, boiled potatoes, and tea or coffee with sugar but no milk or cream) was served. A basal ration which obviously contained only a minimum amount of protein was selected, in order that the hard palates might supply the greater part of the protein of the experimental diet. In making a quantity of the meat loaf sufficient for a three-day digestion experiment for four subjects the following quantities were used: Boiled hard palates finely minced, 13½ pounds; flour, 1 pound; butter, ½ pound; onions, 3 of medium size; and seasoning (sage, salt, and pepper to taste).

METHODS OF DIGESTION EXPERIMENTS

Four subjects who had gained experience in this type of work in the study of the digestibility of other foods assisted in this investigation. They were young men of medium weight and of good health, moderately active, and sufficiently informed through previous experience to appreciate the importance of observing accuracy in following all directions given them.

As is evident from a consideration of their composition and the amounts eaten, hard palates supplied only a small part of the total fat of the experimental ration and a very little ash. Furthermore, since little, if any, carbohydrate was present in the hard palates, it follows that interest centers on the digestibility of protein, since this is the only food constituent which they provide in quantity.

Experience has shown that it is desirable to supply a food constituent in generous proportions in order that the calculated coefficients of digestibility may not be masked by unavoidable errors incidental to the methods followed. To make sure that the amount of protein eaten was generous, a fairly large allowance of the meat loaf made from hard palates was served at each meal and the subjects were urged to eat all of it. At the same time, as already noted above, only a limited amount of protein was obtainable from other sources.

As regards the experimental details, the methods followed in studying the hard palates were similar to those previously reported with other foods.[1] As no attempt was made to maintain body weight or to approximate a nitrogen equilibrium, the quantity of the entire ration to be eaten was not stipulated. The feces occurring from each experimental period, as indicated by charcoal markers, were collected and dried to remove the water. Samples of foods eaten were retained for analysis and all analyses of foods and feces were made by the methods described by the Association of Official Agricultural Chemists.[2]

In order to determine the digestibility of a single food contained in a mixed diet, it is necessary either to determine the digestibility of the basal ration and to apply the proper correction to the values obtained for the digestibility of the total diet, or to estimate the undigested residue occurring from the various constituents of the diet by means of coefficients previously determined, and to make proper allowance for this undigested material. The latter method has been followed in this instance and the method of estimating the digestibility of the protein of the meat loaf alone is indicated by the following equations:

[Weight of protein in potato, crackers, and butter]×[Percentage of undigested protein occurring in each]=[Weight of undigested protein present in feces derived from basal ration].

[Total undigested protein in feces]−[Undigested protein in feces from basal ration]=[Undigested protein occurring from meat loaf].

[(Total protein of meat loaf)−(Undigested protein from meat loaf)]÷[Total protein of meat loaf]=[Estimated percentage digestibility of meat loaf alone].

On the basis of determinations by previous investigators the coefficients assumed in these equations for the digestibility of the protein of the potatoes, crackers, and butter are 83 per cent,[3] 93.8 per cent,[4] and 97 per cent,[3] respectively.

In Table I are recorded the essential experimental data of the digestion experiments with hard palates, including the total weight of food eaten, the nutrients furnished, the weight of feces, the undigested nutrients therein, the percentage of the different nutrients digested, and the estimated digestibility of the protein of the meat loaf.

[1] Langworthy, C. F., and Holmes, A. D. Digestibility of some animal fats. U. S. Dept. Agr. Bul. 125, 22 p. 1915.

[2] Wiley, H. W. Official and provisional methods of analysis, Association of Official Agricultural Chemists. As compiled by the committee on revision of methods. U. S. Dept. Agr. Bur. Chem. Bul. 107 (rev.), 272 p., 13 fig. 1908. Reprinted in 1912.

[3] Atwater, W. O., and Bryant, A. P. The availability and fuel value of food materials. In Conn. Storrs Agr. Exp. Sta. 12th Ann. Rpt., 1899, p. 104. 1900.

[4] Woods, C. D., and Merrill, L. H. Studies on the digestibility and nutritive value of bread at the Maine agricultural experiment station, 1899–1903. U. S. Dept. Agr. Office Exp. Stas. Bul. 143, p. 33. 1904.

TABLE I.—*Results of digestion experiments with hard palates of cattle in a simple mixed diet*

Item.	Weight of food.	Water.	Protein.	Fat.	Carbohydrates.	Ash.
Experiment 334 (subject H. F. B.):						
Hard palates (in form of meat loaf).....gm.	1,309	749.3	317.4	141.2	71.4	29.7
Potato.....gm.	1,242	937.7	31.1	1.2	259.6	12.4
Crackers.....gm.	670	40.0	56.5	96.5	472.3	4.7
Butter.....gm.	261	28.7	2.6	221.9	.....	7.8
Sugar.....gm.	213	.....	.....	.....	213.0	.....
Total food consumed, gm.....	3,695	1,755.7	407.6	460.8	1,016.3	54.6
Feces.....gm.	136	.....	72.3	18.8	32.1	12.8
Amount utilized....gm.	.....	.....	335.3	442.0	984.2	41.8
Digestibility of entire ration.....per cent.	.....	.....	82.3	95.9	96.8	76.6
Estimated digestibility of meat loaf.....per cent.	.....	.....	80.0	.....	.....	.....
Experiment 335 (subject D. G. G.):						
Hard palates (in form of meat loaf).....gm.	1,327	759.6	321.8	143.2	72.3	30.1
Potato.....gm.	1,232	930.2	30.8	1.2	257.5	12.3
Crackers.....gm.	661	39.5	55.8	95.2	465.9	4.6
Butter.....gm.	313	34.4	3.1	266.1	.....	9.4
Sugar.....gm.	111	.....	.....	.....	111.0	.....
Total food consumed, gm.....	3,644	1,763.7	411.5	505.7	906.7	56.4
Feces.....gm.	105	.....	54.5	21.0	20.0	9.5
Amount utilized....gm.	.....	.....	357.0	484.7	886.7	46.9
Digestibility of entire ration.....per cent.	.....	.....	86.8	95.8	97.8	83.2
Estimated digestibility of meat loaf....per cent.	.....	.....	85.8	.....	.....	.....
Experiment 336 (subject R. L. S.):						
Hard palates (in form of meat loaf).....gm.	1,329	760.7	322.3	143.4	72.4	30.2
Potato.....gm.	1,275	962.6	31.9	1.3	266.5	12.7
Crackers.....gm.	291	17.4	24.6	41.9	205.1	2.0
Butter.....gm.	208	22.9	2.1	176.8	.....	6.2
Sugar.....gm.	69	.....	.....	.....	69.0	.....
Total food consumed, gm.....	3,172	1,763.6	380.9	363.4	613.0	51.1
Feces.....gm.	70	.....	32.8	15.9	13.4	7.9
Amount utilized.....gm.	.....	.....	348.1	347.5	599.6	43.2
Digestibility of entire ration.....per cent.	.....	.....	91.4	95.6	97.8	84.5
Estimated digestibility of meat loaf.....per cent.	.....	.....	92.0	.....	.....	.....

TABLE I.—*Results of digestion experiments with hard palates of cattle in a simple mixed diet*—Continued

Item.	Weight of food.	Water.	Protein.	Fat.	Carbohydrates.	Ash.
Experiment 337 (subject O. E. S.):						
Hard palates (in form of meat loaf).....gm..	1,052	602.2	255.1	113.5	57.3	23.9
Potato.....gm..	1,126	850.1	28.2	1.1	235.3	11.3
Crackers.....gm..	525	31.3	44.3	75.6	370.1	3.7
Butter.....gm..	258	28.4	2.6	219.3	.....	7.7
Sugar.....gm..	467	.....	.....	.....	467.0	.....
Total food consumed, gm.....	3,428	1,512.0	330.2	409.5	1,129.7	46.0
Feces.....gm..	98	.....	41.4	31.7	14.7	10.2
Amount utilized.....gm..	.....	.....	288.8	377.8	1,115.0	36.4
Digestibility of entire ration.....per cent..	.....	.....	87.5	92.3	98.7	78.1
Estimated digestibility of meat loaf.....per cent..	.....	.....	86.8	.....	.....	.....
Experiment 342 (subject H. F. B.):						
Hard palates (in form of meat loaf).....gm..	1,312	755.2	336.2	118.5	86.8	15.3
Potato.....gm..	1,216	918.1	30.4	1.2	254.1	12.2
Crackers.....gm..	309	21.3	25.0	41.4	218.8	2.5
Butter.....gm..	107	11.8	1.1	90.9	.....	3.2
Sugar.....gm..	317	.....	.....	.....	317.0	.....
Total food consumed, gm.....	3,261	1,706.4	392.7	252.0	876.7	33.2
Feces.....gm..	97	.....	54.4	14.1	19.9	8.6
Amount utilized.....gm..	.....	.....	338.3	237.9	856.8	24.6
Digestibility of entire ration.....per cent..	.....	.....	86.1	94.4	97.7	74.1
Estimated digestibility of meat loaf.....per cent..	.....	.....	85.8	.....	.....	.....
Experiment 343 (subject D. G. G.):						
Hard palates (in form of meat loaf).....gm..	1,473	847.9	377.5	133.0	97.4	17.2
Potato.....gm..	1,176	887.9	29.4	1.2	245.8	11.7
Crackers.....gm..	376	25.9	30.5	50.4	266.2	3.0
Butter.....gm..	288	31.7	2.9	244.8	.....	8.6
Sugar.....gm..	96	.....	.....	.....	96.0	.....
Total food consumed, gm.....	3,409	1,793.4	440.3	429.4	705.4	40.5
Feces.....gm..	93	.....	50.3	15.5	19.6	7.6
Amount utilized.....gm..	.....	.....	390.0	413.9	685.8	32.9
Digestibility of entire ration.....per cent..	.....	.....	88.6	96.4	97.2	81.2
Estimated digestibility of meat loaf.....per cent..	.....	.....	88.5	.....	.....	.....

TABLE I.—*Results of digestion experiments with hard palates of cattle in a simple mixed diet*—Continued

Item.	Weight of food.	Water.	Protein.	Fat.	Carbohydrates.	Ash.
Experiment 345 (subject O. E. S.):						
Hard palates (in form of meat loaf).....gm..	1,296	746.0	332.1	117.0	85.7	15.2
Potato.....gm..	1,009	761.8	25.2	1.0	210.9	10.1
Crackers.....gm..	345	23.8	27.9	46.2	244.3	2.8
Butter.....gm..	104	11.5	1.0	88.4	.....	3.1
Sugar.....gm..	340	.....	.....	.....	340.0	.....
Total food consumed, gm.....	3,094	1,543.1	386.2	252.6	880.9	31.2
Feces.....gm..	98	.....	45.3	20.4	22.1	10.2
Amount utilized.....gm..	.....	.....	340.9	232.2	858.8	21.0
Digestibility of entire ration.....per cent..	.....	.....	88.2	91.9	97.5	67.3
Estimated digestibility of meat loaf.....per cent..	.....	.....	88.2	.....	.....	.....
Average food consumed per subject per day.gm..	1,129	563.7	130.9	127.3	291.8	14.9

SUMMARY

Experiment No.	Subject.	Digestibility of entire ration (per cent).				Estimated digestibility of protein of meat loaf alone (per cent).
		Protein.	Fat.	Carbohydrates.	Ash.	
334	H. F. B.....	82.3	93.9	96.8	76.6	80.0
335	D. G. C.....	86.8	95.8	97.8	83.2	86.8
336	R. L. S.....	91.4	95.6	97.8	84.5	91.9
337	O. E. S.....	87.5	92.3	98.7	78.1	86.7
342	H. F. B.....	86.1	94.4	97.7	74.1	85.8
343	D. G. G.....	88.6	96.4	97.2	81.2	88.5
345	O. E. S.....	88.2	91.9	97.5	67.3	88.2
	Average.....	87.3	94.6	97.6	77.9	86.8

The average amount of food eaten per subject per day was 1,129 gm. which furnished 564 gm. of water, 131 gm. of protein, 127 gm. of fat, 292 gm. of carbohydrates, and 15 gm. of ash. The uniformity of values obtained in the different experiments for the digestibility of the carbohydrates and the close agreement of the average value, 97.6 per cent, with the value, 97 per cent, given for the digestibility of carbohydrates in the ordinary mixed diet[1] would indicate that care had been observed in the collection of the feces. The digestibility of fat is of interest, in that practically all of the fat of the diet was obtained from the butter,

[1] Atwater, W. O. On the digestibility and availability of food materials. *In* Conn. Storrs Agr. Exp. Sta. 14th Ann. Rpt., 1901, p. 245. 1902.

part of which was present as a constituent of the meat loaf and a part as a constituent of the basal ration, supplying in all approximately 125 gm. of fat per subject per day. This was 94.6 per cent assimilated, which for all practical purposes is identical with the digestibility of butter found in a previous investigation,[1] 93.9 per cent.

Inasmuch as the subjects were allowed to eat of the basal ration according to individual preferences, the energy value of the diet was not uniform. It was found, however, that the subjects eating as much as they wished received, on an average, 3,265 Calories daily, calculated from the average daily consumption of protein, fat, and carbohydrates, and the factors[2] commonly used in the determination of fuel values. In view of the fact that over 130 gm. of protein, largely supplied by the meat loaf, and over 3,200 Calories of energy were consumed daily, it is apparent that the ration was eaten with relish.

The digestibility of the total protein of the diet was found to be 87.3 per cent. The meat loaf supplied 82 per cent of the total protein consumed, a much larger proportion than is ordinarily furnished by the meat portion of a meal; consequently, greater accuracy is possible in estimating the digestibility of the protein contained in the meat loaf.

The digestibility of the protein of the meat loaf alone, 86.8 per cent, differs little from the value of the digestibility of the entire ration. This is due partly to the rather complete assimilation of the protein of the basal ration and partly to the relatively small amount of protein derived from this source. The value, 86.8 per cent, represents the digestible protein of the meat loaf, but it should closely approximate that for the protein of the hard palates, since in the preparation of the loaf the proportions used were 13.5 parts of hard palates to 1 part of flour. An allowance may be made for the flour by assuming the protein from this source to be 93.8 per cent[3] digestible. From the results of this investigation, accordingly, it would seem that the protein of hard palates which have been thoroughly cooked is somewhat less thoroughly assimilated than that of the common cuts of meat.[4]

[1] Langworthy, C. F., and Holmes, A. D. Digestibility of some animal fats. U. S. Dept. Agr. Bul. 312, 22 p. 1915.

[2] Atwater, W. O., and Bryant, A. P. The availability and fuel value of food materials. *In* Conn. Storrs Agr. Exp. Sta. 12th Ann. Rpt. 1899, p. 104. 1900.

[3] Woods, C. D., and Merrill, L. H. Studies on the digestibility and nutritive value of bread at the Maine agricultural experiment station, 1899–1903. U. S. Dept. Agr. Office Exp. Stas. Bul. 143, p. 33. 1904.

[4] Grindley, H. S., Mojonnier, Timothy, and Porter, H. C. Studies of the effect of different methods of cooking upon the thoroughness and ease of digestion of meat at the University of Illinois. U. S. Dept. Agr. Office Exp. Stas. Bul. 193, 100 p. 1907.

SOME PROPERTIES OF THE VIRUS OF THE MOSAIC DISEASE OF TOBACCO

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INTRODUCTION

Several theories have been advanced to explain the physiological origin of the mosaic disease of tobacco (*Nicotiana tabacum*.) Independently, Woods (20) [1] and Heintzel (10) came to the conclusion that oxidizing enzyms are responsible for the disease. Hunger (11) did not accept the enzymic theory of the mosaic disease but considered that unfavorable conditions of growth produced specific toxins within the plant which led to the appearance of the disease. The writer (1) has secured data which do not lend support to the physiological origin of the disease, but indicate that it is dependent upon specific infection.

Further studies of the properties of the expressed sap of mosaic plants, termed the "virus" of the disease, have thrown considerable light on the nature of the infective principle and its relation to some of the enzymic properties of the sap of diseased plants.

Woods (20) and other workers following him have attributed the origin of the mosaic disease to oxidases and peroxidases existing normally in healthy tobacco plants. Since it is a question of fundamental importance to determine whether or not such enzyms are the primary cause of the disease, their relation to infection has been more fully investigated. All data at hand indicate that infection does not depend upon the presence of oxidases or peroxidases, but upon an infective principle which is not a normal constituent of the sap of healthy plants. These conclusions rest upon the fact that methods have been found by which the infective principle may be separated from the oxidases and peroxidases present in the sap of mosaic plants, as shown in the experimental work.

FILTRATION EXPERIMENTS WITH THE VIRUS OF THE DISEASE

FILTRATION THROUGH THE LIVINGSTONE ATMOMETER POROUS CUP

Earlier investigators have shown that the virus of the mosaic disease of tobacco passes through the Berkefeld (normal) filter without losing its infectious properties. The writer's experiments substantiate these results, as shown in Table I, although there is strong indication that the virus becomes attenuated and is less infectious when filtered in this

[1] Reference is made by number to "Literature cited," p. 673-674.

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way. The writer has been unable to obtain the finer pored Berkefeld or Pasteur-Chamberland bougies of the same type because of the European war. By using the Livingstone atmometer porous cup, however, a method of filtration has been devised which has given very interesting results. The construction of this apparatus is shown in Plate XCI. The extracted sap is first filtered through filter paper to remove all suspended material. The clear dark-amber solution is then filtered through the porous cup under reduced pressure (approximately 3 inches of mercury). After passing through the atmometer, the virus has completely lost its infectious properties, yet an intense peroxidase reaction is given with guaiac and hydrogen peroxid.[1]

TABLE I.—*Infectivity of the mosaic virus after it has been filtered through the Livingstone atmometer porous cup in 1915, 10 Connecticut Broadleaf plants having been used in each test*

Virus used.	Enzymic reactions before treatment.	Treatment.	Enzymic reactions after treatment.	Date of inoculation.	Result.
Virus X^{16}	Intense peroxidase, intense catalase.	Untreated	Intense peroxidase, intense catalase.	Nov. 9	8 mosaic.
Do	do	Filtered through paper only.	do	do	Do.
Do	do	Filtered through atmometer.	do	do	All healthy.
Tap water (control).				do	Do.
Virus X^{23}	Intense peroxidase, weak catalase.	Filtered through paper only.	Intense peroxidase, weak catalase.	Nov. 24	4 mosaic.
Virus X^{23}, portion A.	do	Filtered through atmometer, taken after filtering 2 hours.	Intense peroxidase	do	All healthy.
Virus X^{23}, portion B.	do	Filtered through atmometer, taken after filtering 1 hour.	do	do	Do.
Virus X^{23}, portion C.	do	do	do	do	Do.
Virus X^{23}, portion D.	do	do	do	do	Do.
Tap water (control).				do	Do.

FILTRATION THROUGH POWDERED TALC

Numerous experiments have shown that the infective principle of the mosaic disease of tobacco may be completely removed by filtering the virus through powdered talc.

In these experiments (Table II) Hirsch's porcelain funnel, having a diameter of 9 to 10 cm. and furnished with a stationary perforated disk, was used. A disk of hard filter paper was placed over this disk to retain

[1] Woods used the guaiac and guaiac hydrogen-peroxid tests giving the blue coloration for the determination of oxidases and peroxidases in the extracted sap of tobacco plants. Since the oxidase theory as expressed by Woods was based upon results secured with these tests, the same tests were used in the writer's experiments. The terms "intense," "strong," "weak," etc., have been used to designate the relative intensity of the blue coloration. An "intense" peroxidase reaction is one giving at once an intense indigo blue. The term "strong" indicates that the blue coloration is not as deep, and appears more slowly. The term "weak" denotes a light-blue coloration.

the talc, which was mixed with water and poured upon the paper. This filter must be very carefully made, as bubbles and cracks which may form as a result of shrinkage due to drying during the process of making render the results unreliable. Filtration was accomplished by means of a reduced pressure of approximately 3 inches of mercury.

TABLE II.—*Infectivity of mosaic virus after having been filtered through different thicknesses of powdered talc, U. S. P., in 1915*

Virus used.	Peroxidase reactions before filtering.	Material used for inoculation.	Peroxidase reactions after filtering.	Date of inoculation.	Number of plants inoculated.	Results.
X^{16}	Intense	Filtrate from 1-inch talc	Intense	Mar. 6	20	All healthy.
Do	do	Residue on surface of above talc	do	do	10	7 mosaic.
Tap water (control).				do	10	All healthy.
X^{10}	Intense	Filtrate from 1-inch talc	Intense	Mar. 21	20	Do.
Do	do	Residue on surface of above talc	do	do	10	8 mosaic.
Tap water (control).				do	10	All healthy.
X^{9}	Intense	First portion of filtrate from 1¼-inch talc; color light amber.	None	Apr. 29	10	Do.
Do	do	Second portion of above filtrate 2 hours later; color darker.	do	do	20	Do.
Do	do	Residue on surface of above talc	Intense	do	20	19 mosaic.
X^{11}	do	Filtrate from ⅞-inch talc	Strong	do	10	All healthy.
Do	do	do	do	do	10	Do.
Do	do	Filtrate from ¾-inch talc	do	do	10	Do.
Tap water (control).				do	10	Do.
X^{8}	Intense	First portion of filtrate from 1½-inch talc; color light.	None	Apr. 30	10	Do.
Do	do	Second portion of above filtrate; color very dark.	Intense	do	10	Do.
Do	do	Residue on surface of above talc	do	do	10	10 mosaic.
X^{6}	do	First portion of filtrate from 1½-inch talc; color light.	None	do	10	All healthy.
Do	do	Residue on surface of above talc	Strong	do	20	9 mosaic.
X^{26}	do	Filtrate from 1-inch talc	do	May 4	10	All healthy.
Do	do	Residue on surface of above talc	do	do	12	8 mosaic.

Experiments (Table III) have shown that thick layers of talc, by adsorption, remove all the peroxidase from the pure virus. If no peroxidase reactions are shown, or if these reactions have been appreciably weakened, such filtrates have always lost their infectious properties. By reducing the amounts of talc, however, the peroxidase content may be increased until limits are reached beyond which the infective principle also passes into the filtrates. In some of the writer's filtration tests the first portions of the filtrate, giving intense peroxidase reactions, possessed no infectious properties, while the last portions contained the infectious principle of the disease. By using known quantities of powdered talc and constant quantities of different concentrations of virus, it is readily shown that the peroxidase content of the filtrates is not definitely related to infectivity. The Hirsch porcelain funnel was used as in preceding talc filtration tests. The virus was first filtered through paper to remove suspended material. All dilutions were made with distilled water. Filtration was accomplished by means of a reduced pressure of approximately 3 inches of mercury.

TABLE III.—*Infectivity of mosaic virus after filtering constant quantities of different concentrations through weighed amounts of powdered talc, U. S. P.*

Strength of virus after dilution with distilled water.	Peroxidase reactions before filtering.	Quantity of talc.	Time required to filter 50 c. c. of solution.	Peroxidase reactions after filtering.	Number of plants inoculated.	Results.
		Gm.	*Minutes.*			
50 c. c. of undiluted virus	Intense	(a)		Intense	20	15 mosaic.
Do	do	36	30	Weak	10	All healthy.
Do	do	18	20	Intense	10	Do.
50 c. c. of 60 per cent virus	do	18	9	Strong	10	Do.
50 c. c. of 40 per cent virus	do	(a)		do	10	9 mosaic.
Do	Strong	18	6	None	10	All healthy.
50 c. c. of 30 per cent virus	do	18	(b)	do	10	Do.
50 c. c. of 4 per cent virus	do	18	do	do	10	Do.
50 c. c. of 56 per cent virus	Intense	9	9	Strong	10	Do.
50 c. c. of 24 per cent virus	Strong	9	(b)	Weak	10	Do.
50 c. c. of 20 per cent virus	do	9	5	Very weak	10	Do.
50 c. c. of 4 per cent virus	do	9	4	None	10	Do.
50 c. c. of undiluted virus	Intense	9	15	Intense	10	Do.
50 c. c. of 10 per cent virus	Strong	4.5	5	Weak	10	Do.
50 c. c. of 4 per cent virus	do	4.5	5	None	10	Do.
50 c. c. of 5 per cent virus	do	2.3	2	Good	10	Do.
50 c. c. of 3 per cent virus	do	2.3	1¾	None	20	Do.
50 c. c. of 2 per cent virus	Good	1.1	1½	do	10	Do.

[a] Paper only. [b] Not timed.

These results indicate that the infective agents producing the mosaic disease are readily arrested by means of the talc filter. Likewise, it is shown that filtered solutions giving intense peroxidase reactions are no longer infectious.

PRECIPITATION OF THE VIRUS WITH ETHYL ALCOHOL

Experiments have shown that the infective properties of the mosaic disease are quickly destroyed by the higher strengths of ethyl alcohol. Although a strength of 80 per cent appears to destroy the infective properties of the virus in half an hour, the peroxidase continues to give strong reactions with guaiac and hydrogen peroxid. In various experiments the enzyms have been precipitated in solutions of virus of sufficient alcoholic strength to destroy its infective properties. The virus was first passed through filter paper to remove all material in suspension. This gave a clear, dark, wine-colored solution, which was then made up to different alcoholic strengths with absolute alcohol.

In the first test virus X^{16}, giving intense peroxidase and catalase reactions, was used. On November 1, 1915, 200 c. c. of this virus were made up to a 75 per cent alcoholic strength with absolute alcohol. On November 2 the solution was filtered and the precipitate air dried to remove the alcohol. On November 3 the residue remaining was taken up with 50 c. c. of distilled water. Of the filtrate, 750 c. c. were then evaporated to dryness at room temperature, from November 2 to November 4. This filtrate contained neither peroxidase nor catalase. After evaporation, the amber-colored residue, which is readily soluble, was taken up with distilled water.

In a second test the highly infectious virus X^{20}, giving intense peroxidase reactions but no reaction for catalase, was used, and 400 c. c. of this virus

were made up to an alcoholic strength of 80 per cent with absolute alcohol. This solution was prepared on November 6, 1915, and allowed to stand until November 8, when the precipitate was collected by filtration and evaporated to dryness at room temperatures. This residue was taken up with 100 c. c. of distilled water. The original filtrate was also tested for peroxidase and likewise by inoculation.

Since earlier experiments have shown that the infective principle is not destroyed in alcoholic strengths of 45 to 50 per cent for several days, precipitation tests were also made with these strengths. Of virus X^{20}, used in the preceding test, 160 c. c. were made up to a 50 per cent alcoholic solution with absolute alcohol on November 6, 1915. A portion of the supernatant, clear solution was then siphoned off very carefully without disturbing the heavy, flocculent precipitate below. The precipitate was then collected on filter paper and freed from alcohol at room temperatures on November 8 to November 10. This residue was then taken up with 100 c. c. of distilled water (Table IV).

TABLE IV.—*Infectivity of mosaic virus after having been precipitated in 75, 80, 50, and 45 per cent alcoholic solutions*

Virus used.	Alcoholic strengths.	Treatment.	Enzymic reactions after treatment.	Number of plants inoculated.	Result.
	Per cent.				
X^{16}.....	75	Precipitate evaporated dry and taken up with 50 c. c. of water.	Intense peroxidase, intense catalase.	10	All healthy.
Do.....	75	Filtrate from above evaporated dry and taken up with water.	No peroxidase, no catalase.	10	Do.
Do.....	.....	Original, untreated virus.....	Intense peroxidase, intense catalase.	20	17 mosaic.
Tap water only (control).	.....	.....	.....	10	All healthy.
X^{20}.....	80	Precipitate evaporated dry and taken up with 100 c. c. of water.	Intense peroxidase	10	Do.
Do.....	80	Filtrate from above not evaporated.	No peroxidase.....	10	Do.
Do.....	.....	35 c. c. original virus evaporated dry and taken up with 20 c. c. of water.	Intense peroxidase, no catalase.	10	5 mosaic.
Tap water only (control).	.....	.....	.....	10	All healthy.
X^{21}.....	50	Precipitate evaporated dry and taken up with 100 c. c. of water.	Strong peroxidase.	10	9 mosaic.
Do.....	50	Supernatant solution siphoned off from above precipitate and not evaporated.	.....do.....	10	All healthy.
Do.....	45	Precipitate not filtered or evaporated.	Intense peroxidase	10	9 mosaic.
Do.....	45	Second portion of unfiltered precipitate, 15 c. c. diluted with 15 c. c. of water.	.....do.....	10	10 mosaic.
Do.....	45	200 c. c. of precipitate evaporated dry and taken up with 150 c. c. of water.	.....do.....	10	Do.
Do.....	45	Supernatant solution siphoned off, but not filtered or evaporated.	Strong peroxidase.	10	All healthy.
Do.....	45	Supernatant solution siphoned off and filtered through paper only.	.....do.....	10	Do.
Do.....	45	15 c. c. of unfiltered supernatant solution diluted with 15 c. c. of water.	.....do.....	10	Do.
Do.....	45	1,000 c. c. of filtered, supernatant solution evaporated dry and taken up with 400 c. c. of water.	.....do.....	10	Do.
Do.....	.....	Original virus untreated, but diluted to 1 part of virus in 500 parts of water.	No peroxidase.....	10	8 mosaic.
Tap water only (control).	.....	.....	.....	10	All healthy.

In the next experiment a 45 per cent alcoholic solution of virus was made up with virus X^{23} as follows: 825 c. c. of this virus, which had been previously filtered through filter paper, were shaken with 675 c. c. of absolute alcohol on January 14, 1916. On January 15 the clear, supernatant solution was siphoned off. A portion of this was filtered through hard filter paper and a second portion was left unfiltered. Of the unfiltered portion 15 c. c. were also diluted with 15 c. c. of distilled water. On January 15, 1,000 c. c. of the supernatant solution which had been filtered through paper were set aside in a large, shallow dish to evaporate. On January 20 the dry residue was taken up with 400 c. c. of distilled water, which gave a somewhat stronger concentration than the original virus. (See Table IV.)

After decanting off as much of the supernatant solution as possible, the heavy, semiliquid precipitate, or sludge, was treated as follows: A portion was left unfiltered; a second portion was diluted by adding 15 c. c. of distilled water to 15 c. c. of the sludge. In addition to this, 200 c. c. of the sludge were placed in a beaker to evaporate to dryness at room temperatures on January 15. The dry residue was taken up with 150 c. c. of distilled water on January 17. Inoculation tests were now made with the virus after undergoing the various treatments outlined above in connection with precipitations with ethyl alcohol.

From Table IV it will be seen that the infective principle of the virus has been completely destroyed in the 75 per cent and 80 per cent alcoholic solutions, although the precipitates continued to give intense reactions for peroxidase. In these strengths precipitation of the peroxidase was complete, as the supernatant solutions gave no reaction for this enzym.

In the 45 per cent and 50 per cent alcoholic solutions, the infective principle was not appreciably injured. The infective agent, however, appears to have been carried down with the heavy, flocculent precipitates, leaving the supernatant solutions free from infective properties. Owing to the fact that the peroxidase remained in solution, the supernatant solutions continued to give strong peroxidase reactions. According to Chodat and Bach (7), the oxygenase in the sap of a species of Lactarius could be largely precipitated by 40 per cent alcohol, while the peroxidase remained in solution.

The writer's experiments indicate that concentrated solutions of peroxidase precipitated by strong alcohol from the sap of mosaic plants will not produce infection in healthy plants. Furthermore, the writer has carried out successive re-solutions in water and re-precipitations with alcohol in order to obtain purer solutions of peroxidase. Such solutions, however, have never produced infection, although giving intense reactions for peroxidase and in some instances for catalase.

TREATMENT OF THE VIRUS WITH HYDROGEN PEROXID

Experiments have shown that certain quantities of hydrogen peroxid (U. S. P., 3.10 per cent) may be added to the virus of the mosaic disease without destroying its infectious properties. By treating the virus with different quantities of hydrogen peroxid, it is possible to find concentrations which destroy the peroxidase and at the same time leave little or no free hydrogen peroxid in the solution. Schönbein (19, p. 474) and likewise Bach and Chodat (2, p. 603) have observed that while peroxidase activates small amounts of hydrogen peroxid, large amounts of hydrogen peroxid destroy the peroxidase (Table V).

These results show that hydrogen peroxid may destroy the peroxidase in the virus without destroying its infectious properties. Although such solutions no longer give peroxidase reactions, they may retain their infectious properties for a long time. If the quantity of hydrogen peroxid is considerably increased beyond that concentration which is sufficient to destroy all the peroxidase, hydrogen peroxid remains in excess in the solution and the virus sooner or later loses its property of infection.

Chodat (6, p. 642–645) and other investigators have shown the definite relations existing between peroxidase, hydrogen peroxid, and the oxidation products. It has been shown that for constant quantities of peroxidase, the oxidation products increase directly with the amount of hydrogen peroxid present, within certain limits, until all the peroxidase is combined or used up.

The quantity of hydrogen peroxid required to destroy the peroxidase varies greatly, depending upon the composition of the virus. If the virus evolves little or no oxygen upon the addition of hydrogen peroxid, a very small quantity of this reagent destroys the peroxidase.

From Table V it will be seen that a very small quantity of hydrogen peroxid (3.1 per cent, U. S. P.) destroyed the peroxidase in virus X^{20}. As the quantities of hydrogen peroxid were increased, a point was reached where the excess was sufficient to kill the infective principle of the virus. If a considerable excess is present in solutions of virus for any length of time, such solutions lose their green or brown color and become pale or almost as clear as water in some instances. With the addition of 2 c. c. of hydrogen peroxid to 23 c. c. of virus X^{20}, a small excess of hydrogen peroxid was noticeable for several days, but this later disappeared. It has been observed by Bach and Chodat (3, p. 173) that if a mixture of peroxidase and hydrogen peroxid is allowed to stand for some time, both disappear from the solution by mutual interaction and destruction.

TABLE V.—*Effect of hydrogen peroxid upon the infectivity of virus secured from different sources in 1915, 10 plants having been used in each test.*

Virus used.	Date extracted.	Enzymic reactions before treatment.	Treatment.	Enzymic reactions after treatment.	Date inoculated.	Result.
X[8]		Strong peroxidase	15 c. c. virus+5 c. c. H_2O_2, prepared Jan. 2	No peroxidase, Jan. 5	Jan. 5	8 mosaic.
				No peroxidase, May 29	May 29	10 mosaic.
				Fair peroxidase, Jan. 5	Jan. 5	Do.
Do		do	17 c. c. virus+3 c. c. H_2O_2, prepared Jan. 2	Weak peroxidase, Jan. 12		
				No peroxidase, May 29	May 29	3 mosaic.
Do		do	18 c. c. virus+2 c. c. H_2O_2, prepared Jan. 2	Good peroxidase, Jan. 5	Jan. 5	8 mosaic.
Do		do	19.5 c. c. virus+0.5 c. c. H_2O_2, prepared Jan. 2	Strong peroxidase, Jan. 5	do	7 mosaic.
Do		do	Untreated	do	do	10 mosaic.
Tap water (control)					do	All healthy.
X[83]	July 12	Intense peroxidase	5 c. c. virus+15 c. c. H_2O_2, prepared July 13	No peroxidase, July 17	Aug. 21	8 mosaic.
				No peroxidase, Aug. 21		
Do	do	do	8 c. c. virus+12 c. c. H_2O_2, prepared July 13	No peroxidase, July 17	do	Do.
				No peroxidase, Aug. 21		
Do	do	do	10 c. c. virus+10 c. c. H_2O_2, prepared July 13	No peroxidase, July 17	do	Do.
				No peroxidase, Aug. 21		
				No peroxidase, July 17		
Do	do	do	12 c. c. virus+8 c. c. H_2O_2, prepared July 13	No peroxidase, Aug. 21	do	1 mosaic.
				Trace peroxidase, July 17		
Do	do	do	15 c. c. virus+5 c. c. H_2O_2, prepared July 13	No peroxidase, Aug. 21	do	4 mosaic.
				Slight peroxidase, July 17		
Do	do	do	16 c. c. virus+4 c. c. H_2O_2, prepared July 13	No peroxidase, Aug. 21	do	7 mosaic.
				Strong peroxidase, July 17		
Do	do	do	18 c. c. virus+2 c. c. H_2O_2, prepared July 13	Strong peroxidase, Aug. 21	do	4 mosaic.
Do	do	do	Untreated	Intense peroxidase, Aug. 21	do	Do.
Tap water (control)					do	All healthy.
X[98]	Mar. 28	Intense peroxidase, intense catalase.	5 c. c. virus+15 c. c. H_2O_2, prepared July 1	No peroxidase, July 10	Aug. 23	Do.
				No peroxidase, Aug. 23, but H_2O_2 in excess		
				No peroxidase, July 6		
Do	do	do	10 c. c. virus+10 c. c. H_2O_2, prepared July 1	No peroxidase, Aug. 23	do	10 mosaic.
				No H_2O_2 present on Aug. 23		
				Strong peroxidase, July 10		
Do	do	do	15 c. c. virus+5 c. c. H_2O_2, prepared July 1	Good peroxidase, Aug. 23	do	7 mosaic.
Do	do	do	Untreated	Intense peroxidase, Aug. 23	do	5 mosaic.
Tap water (control)					do	All healthy.
X[81]	July 8	Intense peroxidase, strong catalase.	10 c. c. virus+15 c. c. H_2O_2, prepared July 8	No peroxidase, but H_2O_2 in excess Aug. 25	Aug. 25	Do.
Do	do	do	10 c. c. virus+10 c. c. H_2O_2, prepared July 8	No peroxidase, no H_2O_2	July 10	do	9 mosaic.
					Aug. 25		
					Oct. 23	Oct. 23	4 mosaic.
Do	do	do	15 c. c. virus+5 c. c. H_2O_2, prepared July 8	No peroxidase, no H_2O_2	July 10	Aug. 25	7 mosaic.
					Aug. 25		
					July 10		
Do	do	do	17 c. c. virus+3 c. c. H_2O_2 prepared July 8	No peroxidase, no H_2O_2	Aug. 25		8 mosaic.
					Oct. 23	Oct. 23	4 mosaic.

Do	do	do	Untreated	Intense peroxidase, Aug. 25	Aug. 25	9 mosaic.
Tap water (control)	do	do		Strong catalase, Aug. 25	do	All healthy.
				Good peroxidase, Nov. 18		
X[2]	Nov. 1	Intense peroxidase, no catalase.	24 c. c. virus + 1 c. c. H_2O_2 prepared Nov. 17	Very weak peroxidase, Nov. 22	Dec. 21	7 mosaic.
				No peroxidase, Nov. 27		
				No peroxidase, Dec. 20		
				No H_2O_2, Dec. 20		
				Weak peroxidase, Nov. 18		
Do	do	do	23 c. c. virus + 2 c. c. H_2O_2 prepared Nov. 17	No peroxidase, Nov. 22	do	5 mosaic.
				No peroxidase, Nov. 27		
				No H_2O_2 present, Dec. 20		
				No peroxidase, Nov. 18		
Do	do	do	22 c. c. virus + 3 c. c. H_2O_2 prepared Nov. 17	No peroxidase, Dec. 20	do	4 mosaic.
				Much excess H_2O_2, Dec. 20		
				No peroxidase, Nov. 18		
Do	do	do	21 c. c. virus + 4 c. c. H_2O_2 prepared Nov. 17	No peroxidase, Dec. 20	do	All healthy.
				H_2O_2 in excess, Dec. 20		
				No peroxidase, Nov. 18		
Do	do	do	20 c. c. virus + 5 c. c. H_2O_2 prepared Nov. 17	No peroxidase, Dec. 20	do	Do.
				Much excess H_2O_2, Dec. 20		
				No peroxidase, Nov. 18		
Do	do	do	19 c. c. virus + 6 c. c. H_2O_2 prepared Nov. 17	No peroxidase, Dec. 20	do	Do.
				Much excess H_2O_2, Dec. 20		
Do	do	do	Untreated	Intense peroxidase	do	8 mosaic.
Tap water (control)	do	do			do	All healthy.

TREATMENT OF THE VIRUS WITH FORMALDEHYDE

Although formaldehyde destroys the infective principle of the virus in certain concentrations, peroxidase is not appreciably injured for a considerable time at much greater concentrations. Loew (15, p. 29) has shown that the peroxidase of tobacco is unaltered in a 5 per cent solution after 48 hours. It appears that some oxidases also are very resistant to formaldehyde. Kastle (13) found that the oxidase of the mushroom *Lepiota americana* is not destroyed by a 40 per cent formicaldehyde solution which is allowed to act for several days. In the writer's test (Table VI), the peroxidase of tobacco was not appreciably changed in 1 per cent solutions of formaldehyde after standing 30 days. In these tests a 37 per cent U. S. P. solution of formaldehyde was used. All concentrations were made on the assumption that 2.5 c. c. of this solution contained about 1 gm. of formaldehyde. The virus was filtered through paper to remove all suspended material. To subject the virus to a certain strength of formaldehyde, a water solution of formaldehyde just twice as strong as desired for the virus was made up. Equal parts of this solution and the virus were then mixed, thus bringing the formaldehyde strength down to that required for the virus. In this way the virus was uniformly diluted to one-half its original strength in all concentrations of formaldehyde.

TABLE VI.—*Infectivity of the mosaic virus after 31 days' treatment with formaldehyde in 1915, 10 plants having been used in each test*

Virus used.	Enzymic reactions before treatment.	Strength of formaldehyde in the virus solutions.	Date prepared.	Enzymic reactions after treatment.	Date inoculated.	Result.
X[29]	Intense peroxidase.	1 : 100	Nov. 17	Intense peroxidase. Dec. 17.	Dec. 18	All healthy.
Do	do	1 : 200	do	do	do	Do.
Do	do	1 : 400	do	do	do	Do.
Do	do	1 : 600	do	do	do	Do.
Do	do	1 : 800	do	do	do	1 mosaic.
Do	do	1 : 1,000	do	do	do	Do.
Do	do	1 : 1,200	do	do	do	7 mosaic.
Do	do	1 : 1,400	do	do	do	5 mosaic.
Do	do	Untreated	do	do	do	9 mosaic.
Tap water (control).					do	All healthy.

Experiments carried out in 1914 with unfiltered virus treated with the same strengths of formaldehyde and tested 32 days later gave practically the same results. In these tests the virus still retained its infectious properties in that solution which contained 1 part of formaldehyde in 1,000 parts of virus solution. All stronger solutions had lost the power to produce infection.

TREATMENT OF DRIED MOSAIC MATERIAL WITH ETHER, CHLOROFORM, AND OTHER SOLVENTS

In the following experiments dried and ground mosaic material, designated as X^{13}, was used. The original green leaves were harvested on August 31, 1915, and dried in the air. For each solvent the procedure was as follows: Ten gm. of air-dry material were extracted with 70 c. c. of extractive for two days. This solution was then filtered through paper and 35 c. c. of the filtered solution were set aside in a small beaker to evaporate at room temperatures. The residue left after evaporation was brought into 5 c. c. of distilled water and used for inoculation. The original residue, X^{13}, left after filtering off the solvent, was then thoroughly dried at room temperatures and macerated with 50 c. c. of distilled water in a mortar. Ten c. c. of the extract were used for inoculation. In this way the infective properties of the extract and of the original material from which this extract was obtained could be compared.

The process was somewhat different with glycerin, as this is not readily evaporated. After extracting 10 gm. of the dry material for two days the glycerin extract was pressed out and filtered through hard paper under reduced atmospheric pressure. Of the filtered solution 40 c. c. were then made up to 800 c. c. with distilled water, giving a 5 per cent glycerin solution, which will produce no injury when inoculated into tobacco plants. After filtering off the glycerin extract the original X^{13} residue was then subjected to pressure to remove as much glycerin as possible and was then macerated in a mortar with 50 c. c. of distilled water. The results obtained for each solvent are shown in Table VII.

TABLE VII.—*Effect of digestion with ether, chloroform, and other solvents upon infectivity of mosaic material X^{13} dried at room temperatures in 1915, 10 plants having been used in each test*

Solvent used.	Period of extraction.	Time required for extract to evaporate.	Date taken up with water.	Peroxidase reaction after treatment.	Results of inoculating plants Oct. 19, 1915.
Residue X^{13} after digestion with ether.	Oct. 6 to 8...	Oct. 8 to 16...	Oct. 16	Very weak...	8 mosaic.
Ether extract of X^{13}	...do...	Oct. 8 to 12...	Oct. 12	None.....	All healthy.
Residue X^{13} after digestion with chloroform.	...do...	Oct. 8 to 16....	Oct. 16	Very weak...	10 mosaic.
Chloroform extract of X^{13}	...do...	Oct. 8 to 12...	Oct. 12	None.....	All healthy.
Residue X^{13} after digestion with carbon tetrachlorid.	...do...	Oct. 8 to 16....	Oct. 16	Very weak...	5 mosaic.
Carbon tetrachlorid extract of X^{13}.	...do...	Oct. 8 to 12...	Oct. 12	None.....	All healthy.
Residue X^{13} after digestion with toluene.	...do...	Oct. 8 to 16....	Oct. 16	Very weak...	10 mosaic.
Toluene extract of X^{13}	...do...	Oct. 8 to 12...	Oct. 12	None.....	All healthy.
Residue X^{13} after digestion with acetone.	...do...	Oct. 8 to 16....	Oct. 16	Very weak...	8 mosaic.
Acetone extract of X^{13}	...do...	Oct. 8 to 12...	Oct. 12	None.....	All healthy.
Residue X^{13} after digestion with ethyl alcohol.	Oct. 8 to 10...	Oct. 10 to 16....	Oct. 16	Weak.....	Do.
Ethyl alcohol extract of X^{13}	...do...	Oct. 10 to 13...	Oct. 13	None.....	Do.
Residue X^{13} after digestion with methyl alcohol.	...do...	Oct. 10 to 16...	Oct. 16	Very weak...	Do.
Methyl alcohol extract of X^{13}	...do...	Oct. 10 to 15...	Oct. 15	None.....	Do.
Residue X^{13} after digestion with glycerin.	...do...	Not evaporated.	Oct. 16	Very weak...	6 mosaic.
Glycerin extract of X^{13}	...do...	...do.....	Oct. 11	...do.....	10 mosaic.
Residue X^{13} after digestion with water.	...do...	Oct. 10 to 16...	Oct. 16	...do.....	Do.
Water extract of X^{13}	...do...	Not evaporated.	Oct. 12	Good.....	8 mosaic.
Tap water (control)					All healthy.

In the test with glycerin the material to which the glycerin had been added was subjected to maceration and pressure in order to obtain the extract. A later experiment would seem to indicate that if the glycerin extract is poured off without subjecting the residue to maceration or pressure the extract will contain little, if any, of the infectious principle. In this experiment 10 gm. of the same X^{13} material were used. This material, however, was dried over sulphuric acid in a desiccator from November 8 to November 23, 1915. On November 23, 80 c. c. of glycerin were added and allowed to stand until November 27, when the dark-colored extract was merely poured off and filtered through hard paper under reduced atmospheric pressure. Solutions containing 8 and 20 per cent of the extract were made, distilled water being used to dilute the glycerin.

The dry material from which the glycerin extract had been poured off was now washed with 100 c. c. of distilled water. This was poured off and filtered through hard paper, and the material was again washed with 380 c. c. of distilled water. This solution was also poured off and filtered through hard paper. The original leaf material, which was now fairly free from glycerin, was macerated with 25 c. c. of distilled water. The results of testing the above solutions and material for peroxidase and infection are given in Table VIII.

TABLE VIII.—*Results of inoculations with dried material X^{13} digested with glycerin in 1915, 10 plants having been used in each test*

Material used.	Peroxidase reaction after treatment.	Date inoculated.	Result.
8 per cent glycerin extract	No peroxidase (Nov. 27 / Dec. 15)	Dec. 16	1 mosaic.
20 per cent glycerin extract	do	do	All healthy.
100 c. c. water solution (first washing)	do	do	1 mosaic.
380 c. c. water solution (second washing)	do	do	4 mosaic.
Residue X^{13} macerated with 25 c. c. H_2O	Weak peroxidase (Nov. 27 / Dec. 15)	do	10 mosaic.
Tap water only (control)		do	All healthy.

In order to compare the results with dried mosaic material, green mosaic leaf material was also treated with ether, chloroform, and water. For each solvent 25 gm. of finely cut and macerated green mosaic material were used. The quantity of solvent used was about 100 gm.—that is, 140 c. c. of ether, 80 c. c. of chloroform, and 100 c. c. of distilled water. These solvents were added to the green material and shaken on October 20. On October 21 all the solution that could be poured off was then filtered through hard paper and set aside in beakers to evaporate in the air. Of the filtered ether solution 115 c. c., and of the chloroform solution 55 c. c., were obtained. After evaporation, the residues left from the ether and chloroform solutions were each placed in 5 c. c. of distilled water. The leaf material from which these solutions had been obtained

was again dried at room temperatures and macerated with 20 c. c. of distilled water. The results of inoculation experiments with this material are shown in Table IX.

TABLE IX.—*Infectivity of green mosaic leaf material after digestion with ether, chloroform, and water, October 20 and 21, 1915, 10 plants having been used in each test*

Material used.	Time required for extract to evaporate.	Date taken up with water.	Peroxidase reaction after digestion.	Results of inoculating plants Oct. 25, 1915.
Green residue after digestion with ether.	Oct. 21 to 23.....	Oct. 23	Intense peroxidase, Oct. 25..	10 mosaic.
Ether solution from above...	Oct. 21 to 22.....	Oct. 22	Good peroxidase, Oct. 25....	6 mosaic.
Green residue after digestion with chloroform.	Oct. 21 to 23.....	Oct. 23	Intense peroxidase, Oct. 25..	10 mosaic.
Chloroform solution from above.	.....do.....	.....do.	.....do.....	9 mosaic.
Green residue after digestion with water.	.....do.....	.....do...	Good peroxidase, Oct. 25 ...	10 mosaic.
Water solution from above...	Not evaporated.	.....	Intense peroxidase, Oct. 25..	Do.
Tap water (control).....	.....	.....	.....	All healthy.

From Table IX it is evident that the infective principle of the virus was not killed in the ether or chloroform solutions. From similar experiments Clinton (8, p. 415) believed that ether and chloroform could extract the virus from the green leaves to some extent without injury to its infectious properties.

However, from the fact that green crushed material contains a large amount of water, it is very probable that some of this water containing the infective principle passes into the ether or chloroform solutions. Such solutions would represent little more than mixtures of virus and ether, etc. Although the infective principle and likewise peroxidase appeared in the ether and chloroform solutions when green material was used, these did not appear in ether or chloroform extracts made with dry material.

The fact that the infective principle, or even enzyms, appeared in solutions obtained by adding ether, chloroform, toluene, etc., to green material does not justify the conclusion that such substances are soluble in these solvents. Kastle (13, p. 16), working with the oxidases of *Lepiota americana*, found that if toluene is added to portions of the fresh fungus, some of the oxidase passes into the toluene layer. He says:

> Whether the perfectly dry oxidase is soluble in toluene remains to be proved. It may be, of course, that it is the water which is dissolved in the toluene which really takes the oxidase into solution.

Various experiments have shown that the infective principle of the mosaic disease of tobacco is not readily destroyed by ether, chloroform, toluene, or carbon tetrachlorid. Although ether or chloroform vapors quickly kill the green leaf, the infective principle in a mosaic leaf killed in this way remains uninjured after several hours' treatment. Likewise

virus solutions to which several cubic centimeters of ether, chloroform, or toluene have been added did not lose their infectious properties after several months. Carbon tetrachlorid also appears to be quite as inert when added to virus solutions. In a test with this material, 3 c. c. were added to 22 c. c. of virus. The supernatant virus, when tested one month later, was quite as infectious as the untreated.

In other experiments the mosaic sap has been evaporated to dryness at room temperatures in beakers and the residue treated with ether for several days. Under such conditions, however, the residue is only slightly soluble in the ether and there remains a heavy, gummy, more or less impermeable mass. When the ether was evaporated and the residue again taken up with the original amount of water, the solution was still infectious.

Although Clinton (8, p. 415) states that the virus can be preserved for a long time by adding to it a small amount of toluene, the writer's experiments indicate that the virus will retain its infectious properties almost indefinitely without the addition of toluene. With no preservative whatever added, the bottled virus was highly infectious when tested from 12 to 15 months later, although putrefaction had taken place.

TREATMENT OF VIRUS WITH PRECIPITATES OF HYDROXIDS OF ALUMINUM AND NICKEL

Precipitation of the virus of the mosaic disease of tobacco by alcohol in 45 and 50 per cent strengths indicates that the precipitate carries down the infective principle, leaving the supernatant solution without infectious properties. Similar precipitation experiments have been carried out, using aluminum sulphate and nickel sulphate in alkaline solutions of virus to obtain the insoluble hydroxids of these metals. In order to obtain approximately 1 gm. of aluminum hydroxid in the precipitate aluminum sulphate and sodium hydroxid were added according to the following equation:

$$\underset{4.26\text{ gm.}}{Al_2(SO_4)_3.18H_2O} + \underset{1.53\text{ gm.}}{6NaOH} = \underset{2.72\text{ gm.}}{3\ Na_2SO_4} + \underset{1\text{ gm.}}{Al_2(OH)_6} + 18H_2O.$$

The procedure was as follows: On December 1, 1915, 100 c. c. of virus X^{23}, which had been filtered through paper to obtain a clear solution, were made up to 1,000 c. c. with distilled water, thus diluting the virus but 10 times. First, 4.3 gm. of aluminum sulphate dissolved in a small quantity of water were added to the virus solution and shaken. Then 1.5 gm. of sodium hydroxid, dissolved in a small quantity of water, were added and the entire solution shaken and set aside. A very heavy flocculent precipitate of aluminum hydroxid was at once formed. This gradually settled, leaving the supernatant solution perfectly clear. On December 2 the solution was tested with litmus paper and gave a slightly

acid reaction. On this date the greater portion of the clear supernatant solution was carefully siphoned off. After pouring off as much of the remaining supernatant solution as possible, the semiliquid precipitate, or sludge, was bottled. The clear supernatant solution, as well as the sludge, gave intense peroxidase reactions.

As seen from the equation, the treatment of the original virus solution involves the formation of 2.7 gm. of the soluble salt (sodium sulphate) or 1 part in 370 of solution.

As a control to this there was prepared on December 1 a solution of virus of the same dilution as the original, containing 6 gm. of sodium sulphate per 1,000 c. c. of solution, or approximately 1 part of sodium sulphate in 303 parts of solution. It will be noted that this concentration is somewhat higher than that obtained in the reaction to produce 1 gm. of aluminum hydroxid.

The preparation of the nickel-sulphate solution, involving the formation of 2 gm. of nickel hydroxid, was carried out in the same manner as for the aluminum hydroxid. The results of inoculations made with the supernatant solutions and precipitates of aluminum hydroxid and nickel hydroxid are given in Table X.

TABLE X.—*Effect of aluminum hydroxid and nickel hydroxid upon the infectivity of mosaic virus, 10 plants having been used in each test. Material prepared on December 1, 1915*

Material tested.	Enzymic reaction after treatment.	Result of inoculating plants.
Inoculations made on December 16, 1915:		
Semifluid aluminum hydroxid precipitate	Intense peroxidase, Dec. 15, 1915.	1 mosaic.
Do	do	3 mosaic.
Supernatant solution from above precipitate	do	All healthy.
Semifluid nickel hydroxid precipitate	do	Do.
Supernatant solution from above precipitate	do	Do.
Sodium-sulphate-virus solution (1 part sodium sulphate in 303 parts of solution).	do	10 mosaic.
Do	do	Do.
Original virus X^{25} used in above tests, untreated	do	4 mosaic.
Tap water (control)		All healthy.
Inoculations made with above material on January 18, 1916:		
Semifluid aluminum hydroxid precipitate	Intense peroxidase, Jan. 17, 1916.	6 mosaic.
Supernatant solution from above precipitate	do	All healthy.
Semifluid nickel hydroxid precipitate	do	1 mosaic.
Supernatant solution from above precipitate	do	All healthy.
Original virus X^{25} used in above tests, untreated	do	10 mosaic.
Tap water (control)		All healthy.

From the results of Table X it is quite evident that the infective principle of the virus was carried down with the aluminum hydroxid precipitate, leaving the supernatant solution free from infectious properties. Since the treatment with nickel sulphate appears to have destroyed the virus entirely, it is possible that nickel salts are more toxic to the infective principle than the salts of aluminum.

EFFECT OF HEAT UPON THE VIRUS OF THE MOSAIC DISEASE

Several investigators have noted the effect of heat upon the virus of the mosaic disease. Mayer (17, p. 451) found that continued heating at 60° C. did not perceptibly change the infectivity of the virus, but that temperatures of 65° to 75° weakened it. Its infectious properties were completely destroyed when the virus was heated for several hours at 80°.

TABLE XI.—*Effect of heat upon the infectivity of undiluted solutions of mosaic virus heated without previous filtering, in 1915, 10 plants having been used in each test*

Virus used.	Enzymic reaction before treatment.	Date and nature of treatment.	Enzymic reaction after treatment.	Date inoculated.	Results.
X[11]	Intense peroxidas	Heated 15 minutes at 82° C. in test tube suspended in beaker of water, Nov. 23.	Good peroxidase, Nov. 24.	Nov. 24	10 mosaic.
Do.	....do....	Boiled 1 minute in test tube, Nov. 23.	....do....	...do....	All healthy.
Do.	....do....	Boiled 5 minutes in test tube, Nov. 23.	Weak peroxidase, Nov. 24.	...do....	Do.
Do.	....do....	Boiled 10 minutes in test tube, Nov. 23.	....do....	...do....	Do.
Do.	....do....	Unheated....	Intense peroxidase, Nov. 24.	...do....	8 mosaic.
X°	....do....	Heated 10 minutes at 85° C. in test tube suspended in beaker of water, Dec. 2.	Fair peroxidase, Dec. 3.	Dec. 3	9 mosaic.
Do.	....do....	Heated 15 minutes at 85° C., Nov. 23; again heated 7 minutes at 88° C., Dec. 3.	No peroxidase, Dec. 4.	Dec. 4	All healthy.
Do.	....do....	Heated 10 minutes at 90° C. in test tube suspended in beaker of water, Dec. 2.	Weak peroxidase, Dec. 3.	Dec. 3	Do.
Do.	....do....	Heated 10 minutes at 95° C. in test tube suspended in beaker of water, Dec. 2.	Very weak peroxidase, Dec. 3.	...do....	Do.
Do.	....do....	Heated 1 to 2 minutes at 100° C. in test tube suspended in beaker of water, Dec. 2.	Fair peroxidase, Dec. 3.	...do....	Do.
Do.	....do....	Unheated....	Intense peroxidase, Dec. 3.	Dec. 4	8 mosaic.
Control	Strong peroxidase	Tap water and healthy juice, untreated.	Strong peroxidase, Dec. 4.	...do....	All healthy.
X[36]	Intense peroxidase	Heated 5 minutes at 85° C. in test tube suspended in beaker of water, May 8.	Good peroxidase, May 8.	May 10	9 mosaic.
Do.	....do....	Heated 5 minutes at 87° C. in test tube suspended in beaker of water, May 8.	....do....	...do....	8 mosaic.
Do.	....do....	Heated 5 minutes at 88° C. in test tube suspended in beaker of water, May 8.	....do....	...do....	Do.
Do.	....do....	Heated 5 minutes at 90° C. in test tube suspended in beaker of water, May 8.	Fair peroxidase, May 8.	...do....	1 mosaic.
Do.	....do....	Heated 5 minutes at 91° C. in test tube suspended in beaker of water, May 8.	....do....	...do....	All healthy.
Do.	....do....	Heated 5 minutes at 93° C. in test tube suspended in beaker of water, May 8.	....do....	...do....	Do.
Do.	....do....	Heated 5 minutes at 95° C. in test tube suspended in beaker of water, May 8.	Very faint peroxidase, May 8.	...do....	Do.
Do.	....do....	Unheated....	Intense peroxidase, May 8.	...do....	7 mosaic.
Control	Strong peroxidase.	Tap water and healthy juice, untreated.	Strong peroxidase, May 10.	...do....	All healthy.

Iwanowski (12) and, likewise, Beijerinck (4) found that heating the virus to the boiling point destroyed its infectious properties.

According to Koning (14, p. 71–86), who heated the diluted virus in closed tubes, it remained infective when heated 10 minutes at 80°, 5 minutes at 90°, and 5 minutes at 100°.

Woods (20, p. 17–19) believed that the sap of mosaic plants remained infectious to some extent after it had been boiled, owing to the fact that peroxidase was regenerated in the solution.

The writer's experiments indicate that the infective principle of the virus is quickly and permanently destroyed at temperatures near the boiling point, although such solutions may again show good peroxidase reactions. In these tests, in which the test tube containing the virus was suspended in a beaker of heated water, the virus was kept at room temperatures until immersed. The test tube was immersed when the temperature in the beaker had begun to exceed the required point. Owing to the small quantities of virus used, the temperatures in all instances were very quickly brought up to the desired height.

The infective principle of the disease withstands much higher temperatures when the dried mosaic leaf material is subjected to dry heat. In the following experiments the air-dried mosaic leaves were finely ground and dried over sulphuric acid in a desiccator from October 8 to the date of heating. For each test 5 gm. of this powdered material were heated in an electric oven, then macerated and extracted with 25 c. c. of distilled water. The results shown in Table XII were obtained.

TABLE XII.—*Effect of heat upon the infectivity of dried mosaic leaf material in 1915, 10 plants having been used in each test*

Date heated.	Period of heating.	Enzymic reaction after treatment.	Date inoculated.	Results.
Oct. 15	½ hour at 90° C.	Fair peroxidase, Oct. 22	Oct. 23	10 mosaic.
Do.	½ hour at 100° C.	Trace peroxidase, Oct. 29	do.	Do.
Do.	One hour at 100° C.	do.	do.	7 mosaic.
Do.	½ hour at 110° C.	do.	do.	9 mosaic.
Do.	½ hour at 120° C.	No peroxidase, Oct. 22	do.	7 mosaic.
	Dry material, unheated	Fair peroxidase, Oct. 22	do.	10 mosaic.
	Tap water only, unheated		do.	All healthy.
Nov. 5	½ hour at 140° C.	No peroxidase, Nov. 10	Nov. 12	Do.
Do.	½ hour at 150° C.	do.	do.	Do.

Heating experiments were again carried out, using the virus after it had been evaporated to dryness in small beakers. For each test 40 c. c. of undiluted and unfiltered virus X[16] were allowed to evaporate by exposure to the air on October 10, 1915. On November 4 the beakers containing the air-dried residues were heated in an electric oven. After being heated the residues were immediately taken up with 30 c. c. of distilled water.

TABLE XIII.—*Effect of heat upon the infectivity of mosaic virus which has been evaporated to dryness at room temperatures in 1915, 10 plants having been used in each test*

Date heated.	Period of heating.	Enzymic reaction after treatment.	Date inoculated.	Results.
Nov. 4	½ hour at 100° C.	{Fair peroxidase, Nov. 11 {Trace catalase, Nov. 11	Nov. 12	All healthy.
Do.	½ hour at 110° C.	{No peroxidase, Nov. 11 {No catalase, Nov. 11	do.	Do.
Do.	½ hour at 120° C.	do.	do.	Do.
Do.	½ hour at 130° C.	do.	do.	Do.
	Original virus evaporated air-dry, but not heated.	{Weak peroxidase, Nov. 11 {Intense catalase, Nov. 11	do.	5 mosaic.

From the data in Table XIII it is evident that the evaporated virus solution lost its infectious properties much more quickly than the dried and ground mosaic leaf material. Likewise, the peroxidase was somewhat more quickly destroyed in the evaporated material. Although the presence of small amounts of moisture in the air-dried residue of the evaporated virus may have hastened the destruction of the infective principle in this material, it is also possible that the infective principle is better able to withstand high temperatures when allowed to remain within the tissues of the leaf.

Although the virus with which Koning (14, p. 71–86) worked appears to have withstood temperatures as high as 100° C., the virus with which the writer worked is very quickly destroyed at temperatures above 90°. In some instances, however, the virus has been rendered noninfectious at a temperature 10 degrees lower than this. These results were obtained with the virus designated as X^{16}. This virus was extracted from tobacco plants on September 20, 1915, and bottled until used on October 18, 1915. Although the unheated virus was highly infectious, the infective principle was destroyed after heating for 5 minutes at 80°. It was also destroyed when heated for 2 minutes at 81°. Although the original, unheated virus gave intense reactions for catalase and peroxidase, the catalase was completely destroyed in these tests. Weak peroxidase reactions, however, were again shown the next day.

As Woods (20) has shown, there is frequently a return of peroxidase activity in solutions of virus that have been once heated. This activity does not appear to return immediately after cooling, but usually requires some hours for its return. Woods considered that the enzyn was destroyed in such solutions but a resistant zymogen again generated more of the peroxidase after cooling. On the other hand, Hasselbring and Alsberg (9) were led to believe from their experiments that a zymogen might not be present, but that the enzym was included and protected in the coagulum and subsequently leached out on standing.

By heating the virus several times at 85° C., the writer has been able in some instances to destroy completely the peroxidase present without destroying the infective principle. The highly infectious virus designated as X° and showing intense peroxidase reactions was treated as

follows: Heated 10 minutes at 85° on December 2, 1914. Cooled at once. A fair peroxidase reaction shown on December 3. Again heated 10 minutes at 85° on December 4, and cooled at once. Weak peroxidase reaction shown December 5. Again heated 10 minutes at 85° on December 5 and immediately cooled. Very weak peroxidase reaction was shown on December 16, when it was again heated for the fourth time for 15 minutes at 85°. When used for inoculation on December 28, no peroxidase reaction was shown. The virus was still highly infectious, however, and produced the mosaic disease in 9 out of 10 plants.

EFFECT OF LOW TEMPERATURES UPON THE VIRUS

After having been frozen for periods varying from one to four hours at −12° C., the extracted sap of mosaic plants still retained its infectious properties unchanged. It likewise retained its original virulence after having been exposed outdoors during the entire winter of 1915 and allowed to freeze and thaw repeatedly. In recent experiments liquid air was used to freeze the virus, and a temperature of approximately −180° was reached. The results are given in Table XIV.

TABLE XIV.—*Effect upon infectivity of freezing fresh mosaic sap to −180° C. by means of liquid air in 1916, 10 plants having been used in each test*

Material used.	Time exposed to liquid air.	Peroxidase reaction before freezing.	Peroxidase reaction after freezing.	Result of inoculating plants, Feb. 2, 1916.
	Minutes.			
Original virus, unfrozen		Intense, Feb. 1		10 mosaic.
Original virus, frozen Jan. 31	15	do	Intense, Feb. 1	Do.
Duplicate of above	15	do	do	Do.
Tap water and healthy sap, unfrozen		do		All healthy.

These tests indicate that the infective principle of the mosaic disease of tobacco is highly resistant to extremely low temperatures.

DISAPPEARANCE OF PEROXIDASE IN MOSAIC VIRUS WITHOUT LOSS OF INFECTIOUS PROPERTIES

It has been observed in several instances that unpreserved solutions of virus, as well as dried and ground mosaic material, may lose their peroxidase activities and still retain infectious properties. This happened with dried and ground mosaic leaves bottled in December, 1912. This material showed fair peroxidase reactions on January 28, 1915, but no reactions for peroxidase in October, 1915. At this time the virus still retained the power to produce infection.

In another instance a bottle of unpreserved virus which was extracted on April 27, 1914, failed to give peroxidase reactions on December 3, 1914; yet at this time was highly infectious, producing the disease in 9 plants out of 10 inoculated. This virus was also highly infectious when tested on May 15, 1915, producing the mosaic disease in 8 out of 10 plants. Although the virus was not tested for peroxidase at the time it was extracted, the fresh virus would probably have shown peroxidase

reactions. In the writer's experience, freshly extracted sap from healthy plants as well as from plants affected with the mosaic disease has never failed to give more or less intense peroxidase reactions.

Various experiments have shown that talc-treated virus slowly loses its peroxidase activities, although still retaining its infectious properties, as shown in the following test: On November 19, 1915, 50 c. c. of virus X^{20}, extracted on November 1, 1915, and filtered through paper, were mixed to a thick paste with 72 gm. of powdered talc, U. S. P. This material was tested for peroxidase reaction and infectivity from time to time with the following results:

Strong peroxidase reaction	Nov. 19, 1915.
Very weak peroxidase reaction	Nov. 30, 1915.
Very faint peroxidase reaction	Dec. 20, 1915.... 10 plants mosaic.
No peroxidase reaction	Jan. 18, 1916.... 4 plants mosaic.

Similar results have been noted when the virus, and also green mosaic material, have been buried in the soil. The virus, and likewise the green material, may entirely lose their peroxidase activities on decaying, although still retaining the power to produce infection.

INFECTIOUS PROPERTIES LOST AND PEROXIDASE ACTIVITIES RETAINED

While the infective principle of the mosaic disease appears to be very resistant, the infectivity of a virus solution may be lost under some conditions, although the peroxidase is not appreciably changed. This was noted as a result of evaporating a quantity of virus to dryness. A solution of 350 c. c. of virus which had been extracted some time previously and allowed to undergo free fermentation was evaporated at room temperature from September 18 to October 21, 1915. On October 21 the solution had been reduced to 20 c. c. of a thick, heavy, putrid-smelling black sirup. Although this solution gave much more intense reactions for catalase and peroxidase than the original solution, showing that these enzyms had been concentrated during the process of evaporation, the infective principle of the virus had been completely destroyed. Inoculation tests showed that the original virus, however, still retained its infectious properties.

Although in this instance the infective principle had been destroyed, many tests have shown that the virus of the mosaic disease is not usually destroyed, even when evaporated to dryness.

Experiments with the feces of hornworms fed upon the leaves of mosaic plants have given rather interesting results. After the worms had been feeding upon the plants for a day or two the feces were collected and macerated with distilled water. In one test the feces of a single worm were used. Out of 10 plants inoculated, one plant only became diseased. Since but one case of the mosaic disease appeared in this test, there is a possibility that this plant developed the disease as a result of accidental infection from other sources.

In another experiment six hornworms which had been feeding upon mosaic plants in the field were transferred to mosaic plants in the laboratory and left for a day or two. The feces were then collected, macerated with tap water, and tested as follows: Ten plants were inoculated with the extracted sap of mosaic leaves upon which the worms were allowed to feed. Nine plants became mosaic. Ten plants were inoculated with a water extract of the feces of the hornworms. All remained healthy. Ten plants were inoculated with tap water (control). All remained healthy.

Although these results indicate that the infective principle of the original material had been destroyed by the digestive process of the worms, the feces gave intense peroxidase reactions.

INFECTIVE PRINCIPLE OF THE DISEASE NOT A NORMAL CONSTITUENT OF THE SAP OF HEALTHY PLANTS

Woods, from his cutting-back experiments with tobacco and other plants, was led to believe that the mosaic disease of tobacco had its origin within the cells of the plants as a result of abnormal physiological activities. Although Woods ascribed the origin of the disease to peroxidase, he believed that there was no essential difference between the peroxidase of healthy and that of diseased plants and came to the conclusion that this enzym obtained from either source could produce the disease.

In an earlier paper (1) the writer has adduced evidence to show that the disease is not produced by simply cutting back or otherwise subjecting plants to unfavorable conditions. In the present paper it has also been shown that peroxidase bears no essential relation to infection and that by various methods this and other enzyms may be more or less completely removed from the virus without affecting the infective principle of the disease, and vice versa.

Although the sap of healthy plants may be rich in oxidase, peroxidase, and catalase, such sap never produces the mosaic disease in healthy plants. Although the peroxidase of diseased plants may be decreased to such an extent by dilution with distilled water that it can not be detected by the guaiac-hydrogen-peroxid test, the solution still remains highly infectious. The results of the experiments in which the virus was diluted with distilled water make this plain (Table XV).

TABLE XV.—*Effect of dilution of mosaic virus with distilled water*

Degree of dilution.	Peroxidase reaction.	Number of plants inoculated.	Result.
Virus undiluted	Intense		
1 part virus in 250 parts water	Weak		
1 part virus in 500 parts water	None	10	8 mosaic.
1 part virus in 1,000 parts water	do	10	6 mosaic.
Tap water only (control)		10	All healthy.

On the other hand, by evaporation the enzyms present in the sap of healthy plants may be brought to the highest possible concentration, but such solutions never acquire infectious properties.

That oxidase (producing the blue color with guaiac alone) can not be responsible for the mosaic disease may be shown by heating the solution to 70° C. for several minutes. This temperature destroys the oxidase, according to Loew (15, p. 31), but does not affect the peroxidase or the principle of infection. As a matter of fact, the oxidase of the tobacco sap appears to be an unstable enzym and very soon disappears entirely from untreated solutions on standing.

Although the enzym termed "catalase" by Loew is very often a normal constituent of healthy and mosaic plants, it can be shown that the presence of this enzym has nothing to do with infection. As shown by Loew (16, p. 19), catalase is destroyed by heating the solution for a minute or two at 80° C. Such solutions, although no longer showing reactions for catalase, may yet retain their infectious properties.

Although it is known that other enzyms than oxidase, peroxidase, and catalase occur normally in the sap of healthy tobacco plants (18), such enzyms can not be considered in a causal relationship to the mosaic disease if it has been established that this disease is not of so-called physiological or spontaneous origin—that is, it can not occur in the absence of infection. Furthermore, the writer sees no reason to believe that any specific enzym occurs in a mosaic tobacco plant which would not be found in healthy plants.

Although it has been shown by various workers that the enzymic relations and reactions in plants become disturbed as a result of disease and unfavorable conditions of growth, there is no reason to believe that these disturbances, when associated with the mosaic disease of tobacco, hold a causal relation to the disease. It is now well known that various factors, aside from pathological conditions caused by an unknown infective principle, may change the quantitative relations of enzyms in plants, as Bunzel (5) has shown in studying the curly-top of sugar beets. It yet remains to be shown that an increase in the amount or activity of enzyms in diseased plants is anything more than a symptom or an indication of disturbed metabolism as a result of the disease.

In the writer's experience all evidence at hand indicates that the mosaic disease of tobacco is dependent upon a specific pathogenic agent which must be introduced into healthy plants from without before the disease can arise. That this pathogenic entity is highly infectious and is in some manner reproduced within the plant are established facts. If these facts are interpreted according to those fundamental principles upon which all our scientific conceptions in pathology and biology are based, that infectious diseases are associated with parasitism and that self-reproduction is a characteristic of living things alone, it must be admitted that the pathogenic agents responsible for the mosaic disease of tobacco must be parasites. If from the facts stated above it is held that nonliving chemical substances such as enzyms or toxins engender the disease, our fundamental biological conceptions no longer hold true.

SUMMARY

In this paper are given the results of a study of the properties of the virus of the mosaic disease of tobacco, and evidence is adduced to show that the infective principle can not be identified with peroxidase. Briefly, the facts obtained may be stated as follows:

(1) The infective principle of the mosaic disease of tobacco is retained by the Livingstone atmometer porous cup used as a filter, and also by powdered talc. Although the filtrates may show intense peroxidase reactions, they no longer produce infection.

(2) The infective principle of the disease is quickly destroyed in alcohol of a strength of 75 to 80 per cent. In this strength precipitation of the peroxidase is complete. By filtering the solution the peroxidase may be collected, freed from alcohol by evaporation, and redissolved with water. This solution gives intense peroxidase reactions, but no longer produces infection. Alcoholic solutions of virus of 45 and 50 per cent strengths did not destroy the infective principle of the disease within the same period. In these solutions the pathogenic agents are not destroyed and appear to be carried down with the precipitate, leaving the supernatant solution without infectious properties, although giving strong peroxidase reactions.

(3) By the addition of different quantities of hydrogen peroxid to the virus, it is possible to find a concentration of sufficient strength to destroy the peroxidase, but leaving little or no free peroxid in the solution. Such solutions no longer show peroxidase reactions, but retain their infectious properties for a long time. A considerable excess of hydrogen peroxid destroys the infective principle itself. The quantity of hydrogen peroxid required to destroy the peroxidase without leaving any considerable excess in the solution depends upon the nature of the virus, the amount of active catalase present, etc.

(4) The virus was treated with formaldehyde for 31 days in the following concentrations: One part formaldehyde in 100, 200, 400, 600, 800, 1,000, 1,200, and 1,500 parts of virus solution. The solutions containing 1 part formaldehyde in 800, 1,000, 1,200, and 1,500 parts of solution gave infection. Stronger concentrations were no longer infectious, although giving intense reactions for peroxidase.

(5) Ether, chloroform, carbon tetrachlorid, toluene, and acetone failed to extract either the infective principle or the peroxidase from dried mosaic material. These solvents also failed to destroy the infectious principle in this material. Ethyl and methyl alcohol completely destroyed the infective principle in the leaf material itself, as well as in the extract. No evidence of peroxidase was obtained in the alcohol extracts. Glycerin does not destroy the infective principle of the disease. Water extracts of dried material not only show peroxidase reactions, but also contain the infective principle of the disease.

(6) A precipitate of aluminum hydroxid, formed by adding aluminum sulphate to alkaline solutions of virus, appeared to carry down the infec-

tive principle of the disease, leaving the clear, supernatant solution without infectious properties, although showing good peroxidase reactions. Similar treatment with nickel sulphate was not so satisfactory, as it gave evidence of being more toxic to the infective principle than the aluminum salt.

(7) The virus is quickly killed at temperatures near the boiling point of water. In some instances heating the virus for five minutes at 80° C. was sufficient to destroy its infectivity. In other tests, with a different virus solution, heating for five minutes at 90° did not entirely destroy its infectivity. In dried and ground mosaic material, rendered water-free by drying over sulphuric acid in a desiccator, the infective principle resisted much higher temperatures than it did in solutions. If virus solutions are heated, the thermal death point of the infective principle is lower than that of the peroxidase; or at least it is more quickly destroyed than the peroxidase.

(8) The virus is highly resistant to low temperatures. When frozen to a temperature of −180° C. with liquid air, its infectious properties were not weakened.

(9) Unpreserved solutions of virus have sometimes lost their peroxidase activities without losing their infectious properties. Dried and ground mosaic material has also lost its peroxidase activities and still remained highly infectious. Talc-treated material, while retaining its infectious properties, has lost its peroxidase activities.

(10) Solutions of virus sometimes lose their infectious properties and continue to show intense peroxidase reactions, as when allowed to evaporate spontaneously in one instance. The feces of worms fed upon mosaic plants have, in some instances, failed to produce infection, although such material continued to give intense peroxidase reactions.

(11) The writer's experiments show that peroxidase or catalase in the sap of mosaic plants can not be responsible for the mosaic disease. The same enzyms are normally present in healthy plants, but the sap of such plants is without infectious properties. By evaporation the enzyms present in healthy sap may be brought to a high concentration, and such solutions never acquire infectious properties. By dilution, on the other hand, the peroxidase content of mosaic sap may be diminished to such an extent that peroxidase reactions are no longer discernible; yet such solutions may remain highly infectious.

Since it has been shown that the mosaic disease of tobacco does not occur in the absence of infection, neither enzyms nor other normal constituents in the sap of healthy plants can be considered responsible for the disease. A specific, particulate substance not a normal constituent of healthy plants is the cause of the disease. Since this pathogenic agent is highly infectious and is capable of increasing indefinitely within susceptible plants, there is every reason to believe that it is an ultramicroscopic parasite of some kind.

LITERATURE CITED

(1) ALLARD, H. A.
1914. The mosaic disease of tobacco. U. S. Dept. Agr. Bul. 40, 33 p., 7 pl.

(2) BACH, A., and CHODAT, R.
1903. Untersuchungen über die Rolle der Peroxyde in der Chemie der lebenden Zelle. IV. Ueber Peroxydase. *In* Ber. Deut. Chem. Gesell., Jahrg. 36, No. 3, p. 600–605.

(3) ——— ———
1908. Die Oxydationsvorgänge in der lebenden Zelle. *In* Centbl. Agr. Chem., Jahrg. 37, Heft 3, p. 168–177.

(4) BEIJERINCK, M. W.
1898. Ueber ein Contagium vivum fluidum als Ursache der Fleckenkrankheit der Tabaksblätter. *In* Verhandel. Akad. Wetensch. Amsterdam, sect. 2, deel 6, no. 5, 22 p., 2 pl.

(5) BUNZEL, H. H.
1913. Biochemical study of the curly-top of sugar beets. U. S. Dept. Agr. Bur. Plant Indus. Bul. 277, 28 p. Literature cited, p. 28.

(6) CHODAT, R.
1905. Les ferments oxydants. *In* Schweiz. Wchnschr. Chem. u. Pharm., Jahrg. 43, No. 47, p. 642–645; No. 48, p. 655–659.

(7) ——— and BACH, A.
1903. Untersuchungen über die Rolle der Peroxyde in der Chemie der lebenden Zelle. V. Zerlegung der sogenannten Oxydasen in Oxygenasen und Peroxydasen. *In* Ber. Deut. Chem. Gesell., Jahrg. 36, Heft 3, p. 606–608.

(8) CLINTON, G. P.
1915. Chlorosis of plants with special reference to calico of tobacco. *In* Conn. Agr. Exp. Sta. Rpt. 1914, p. 357–424, pl. 25–32.

(9) HASSELBRING, Heinrich, and ALSBERG, C. L.
1910. Studies upon oxidases. (Abstract.) *In* Science, n. s. v. 31, no. 799, p. 637.

(10) HEINTZEL, Kurt.
1900. Contagiöse Pflanzenkrankheiten ohne Microben, mit besonderer Berücksichtigung der Mosaikkrankheit der Tabaksblätter. 46 p., 1 pl. Erlangen. Inaugural-Dissertation.

(11) HUNGER, F. W. T.
1905. Untersuchungen und Betrachtungen über die Mosaikkrankheit der Tabakspflanze. *In* Ztschr. Pflanzenkrank., Bd. 15, No. 5, p. 257–311.

(12) IWANOWSKI, D.
1892. Ueber zwei Krankheiten der Tabakspflanze. (Abstract.) *In* Beih. Bot. Centbl., Jahrg. 3, p. 266–268. 1893. Original in Land- u. Forstw. [Russisch] 1892. Not seen.

(13) KASTLE, J. H.
1906. On the stability of the oxidases and their conduct toward various reagents. *In* Publ. Health and Mar. Hosp. Serv. U. S., Hyg. Lab. Bul. 26, p. 7–22.

(14) KONING, C. J.
1900. Der Tabak. Studien über seine Kultur und Biologie. 86 p., 15 fig. Amsterdam, Leipzig.

(15) LOEW, Oscar.
1900. Physiological studies of Connecticut leaf tobacco. U. S. Dept. Agr. Rpt. 65, 57 p.

(16) LOEW, Oscar.
1901. Catalase, a new enzym of general occurrence, with special reference to the tobacco plant. U. S. Dept. Agr. Rpt. 68, 47 p.
(17) MAYER, Adolf.
1886. Ueber die Mosaikkrankheit des Tabaks. *In* Landw. Vers. Stat., Bd. 32, p. 450–467, pl. 3.
(18) OOSTHUIZEN, J. du P., and SHEDD, O. M.
1913. The enzymes of the tobacco plant. *In* Jour. Amer. Chem. Soc., v. 35, no. 9, p. 1289–1309.
(19) SCHÖNBEIN, C. F.
1857. Ueber chemische Berührungswirkungen. *In* Verhandl. Naturf. Gesell. Basel, T. 1, p. 467–482.
(20) WOODS, A. F.
1902. Observations on the mosaic disease of tobacco. U. S. Dept. Agr. Bur. Plant Indus. Bul. 18, 24 p., 6 pl.

PLATE XCI

Livingstone atmometer porus cup as used for filtration. The virus of the mosaic disease of tobacco always lost its infectious properties in passing through this filter.

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